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SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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RELATION OF VARICELLA TO HERPES ZOSTER.

I. Statistical Observations.

By T. M. RIVERS, M.D., and L. A. ELDRIDGE, Jr., M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, February 13, 1929.)

Varicella, herpes simplex, and herpes zoster are diseases characterized by vesicular eruptions in the skin. Macroscopically and microscopically the individual lesions (1–4) in these maladies are frequently very similar. In spite of the striking similarity exhibited by the individual lesions, the picture as a whole, *i.e.*, distribution of the rash and immune reactions, is as a rule characteristic for each disease.

It is now generally believed that varicella and varioloid are caused by different viruses. Such a remark at present is trite, but a hundred years ago the difference between the diseases was questioned by many observers, and certain workers (5), even 30 years after Jenner's report on vaccination, still thought that chicken-pox and smallpox were produced by the same infectious agent.

Herpes simplex is so frequently associated with other diseases, *e.g.*, pneumonia, meningitis, malaria, that the name of symptomatic herpes is often used in referring to it. Through the recent work of Grüter (6), Löwenstein (7), and others, it is now known that this type of herpes, occurring alone or in connection with other diseases, is caused by a specific virus. As soon as it was admitted to the virus group, a discussion arose regarding its relation to herpes zoster. From Cole and Kuttner's (8) review of the literature and from their work concerning the identity of the diseases, it seems unlikely that the majority of cases of herpes simplex and herpes zoster are caused by the same virus.

Herpes zoster, because of its frequent association with injury of nerves and spinal ganglia, for a long time (1861) was considered to be a manifestation of trophic disturbances in the skin attendant upon altered innervation. This conception, however, of the etiology was not applicable to all cases of zoster, and soon (1878) certain cases came to be looked upon as being caused by an infectious agent. In 1900, Head and Campbell (9) reviewed the literature and presented their work upon the subject. They concluded, because of the changes observed in the spinal ganglia and nerves, in the skin, and in the lymph nodes, that many of their cases of zoster were produced by a specific virus. Other cases, however, were thought to be caused not by a virus but by a mechanical or chemical irritation of spinal ganglia.

In 1909 Bókay (10) set forth a hypothesis, based on clinical observations, that some cases of zoster (herpes zoster varicellosa) are caused by the virus of chicken-pox and that such cases are capable of giving rise to varicella in exposed susceptible individuals. Following Bókay's report, numerous observations confirming or refuting his idea have been recorded. Most of the reports are similar to those made a hundred years ago in regard to the relation of chicken-pox to varioloid and smallpox and consist in recording the occurrence of varicella in individuals exposed to zoster and *vice versa* or in relating the coexistence of the two diseases in the same person or in individuals in the same family. Chicken-pox is a common disease and zoster is not rare (11), consequently reports of the nature above mentioned are of little value, inasmuch as coincidence alone doubtless accounts for the apparent relation of the two diseases in certain instances. There are reports, however, of a different nature that must be considered seriously. These will be discussed in the second paper of this series.

The real problem regarding the diseases under discussion is not whether the virus of herpes simplex under rare conditions can cause a disease indistinguishable from zoster, or whether the virus of varicella under the proper circumstances is capable of producing a malady similar to zoster and *vice versa*, but whether there are 3 distinct viruses, though they be very much alike in certain respects, each of which as a rule causing a distinct clinical picture, herpes simplex, herpes zoster, and varicella. If the same virus is instrumental in producing the majority of the cases of any 2 of the diseases, certain types of statistical data should reveal this fact. The purpose of the present paper is to present such data that seems to bear upon the identity of the causal agents of the majority of cases of herpes zoster and varicella.

Methods and Materials.

Herpes Zoster.—The records of cases of herpes zoster at the Bellevue and Vanderbilt clinics were examined and from them information concerning the monthly incidence and the age incidence of the disease was obtained. The selected clinics are large and their records undoubtedly are good indices of the prevalence of zoster at any given time throughout New York City.

Varicella.—The data concerning the monthly incidence of chicken-pox in New York City from Jan., 1914, through July, 1926, and from Aug., 1926, through Dec., 1926, were obtained respectively from the Monthly and the Weekly Bulletins of the Department of Health of the City of New York. The information regarding the age incidence of varicella was derived from the records of cases of chicken-pox observed at the Rockefeller Hospital, 1922–28.

TABLE I.

Tabulation of the Number of Cases of Varicella Reported Each Month for 13 Years in New York City.

Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total
1914	1,121	1,156	1,222	1,217	1,451	1,104	319	85	56	315	574	1,112	9,732
1915	1,341	1,134	1,423	1,073	989	1,220	277	89	77	149	288	661	8,701
1916	956	874	1,439	1,623	1,747	1,331	430	70	44	303	325	631	9,779
1917	874	876	1,294	957	1,212	1,183	217	78	64	141	584	906	8,386
1918	785	598	559	441	542	363	175	95	56	136	174	272	3,776
1919	414	412	599	693	963	786	187	67	63	169	287	686	5,326
1920	726	492	536	467	629	680	227	80	74	230	485	908	5,534
1921	1,212	1,166	1,311	924	939	806	263	49	59	119	410	683	7,941
1922	841	656	663	540	673	490	131	48	67	207	475	947	5,738
1923	1,289	997	780	785	1,235	1,259	398	134	65	195	538	996	8,471
1924	1,155	1,048	1,200	1,000	857	767	230	108	83	318	636	858	8,260
1925	845	864	909	798	871	1,211	250	86	68	164	588	892	7,646
1926	1,091	874	645	456	542	731	343	111	57	241	650	963	6,704
Total.....	12,650	11,147	12,580	10,974	12,650	11,951	3,447	1,100	833	2,687	6,014	10,521	95,994
Mean 1914–26.	973	857	967	844	973	919	265	84	64	206	462	809	7,384
Mean 1922–26.	1,044	708	839	716	836	892	270	97	78	225	577	931	

TABLE II.

Tabulation of the Number of Cases of Herpes Zoster Observed Each Month for 13 Years at the Bellevue Clinic.

Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Male	Female
1914	2	2	1	5	1	3	2	3	1	2	1	2	14	11
1915	3	0	5	0	2	4	1	2	4	3	2	2	14	14
1916	0	1	2	2	2	0	3	1	1	1	2	4	11	8
1917	1	2	1	3	2	0	0	2	4	2	2	2	12	9
1918	6	0	2	0	2	4	2	1	3	2	2	0	15	9
1919	4	5	5	1	2	4	0	2	1	4	0	3	19	12
1920	2	3	3	2	3	3	0	4	3	3	2	3	19	12
1921	2	3	5	2	1	3	5	4	3	3	4	2	24	13
1922	8	5	2	3	7	2	4	2	4	3	7	1	35	13
1923	4	3	2	7	7	5	4	2	2	4	1	5	32	14
1924	2	2	3	3	4	4	3	2	3	6	4	3	30	9
1925	5	3	4	3	3	5	2	3	5	0	2	3	31	7
1926	4	5	0	2	5	3	4	6	4	0	1	4	24	14
Total.....	43	34	35	33	41	40	30	34	38	33	30	34	280	145
Mean 1914–26..	3.3	2.6	2.7	2.5	3.2	3.1	2.3	2.6	2.9	2.5	2.3	2.6		
Mean 1922–26..	4.6	3.6	2.2	3.6	5.2	3.8	3.4	3.0	3.6	2.6	3.0	3.2		

TABLE III.

Tabulation of the Number of Cases of Herpes Zoster Observed Each Month for 5 Years at the Vanderbilt Clinic.

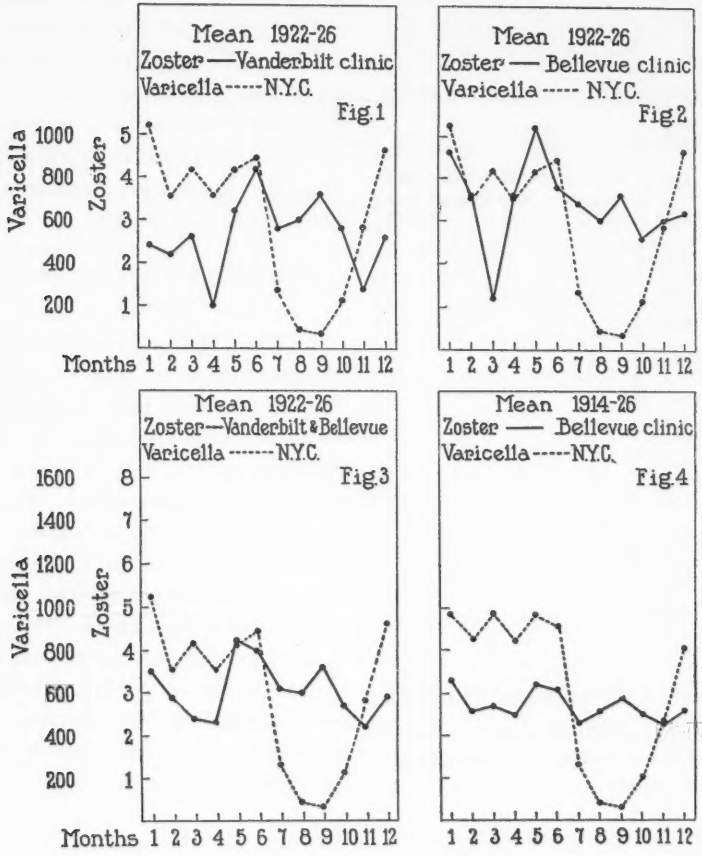
Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
1922	3	2	3	0	3	5	1	4	1	1	3	2
1923	1	3	2	1	5	5	3	5	2	6	2	5
1924	4	2	2	0	2	5	5	5	5	1	0	0
1925	3	4	3	3	2	2	2	1	8	1	1	2
1926	1	0	3	1	4	4	3	0	2	5	1	4
Total...	12	11	13	5	16	21	14	15	18	14	7	13
Mean...	2.4	2.2	2.6	1	3.2	4.2	2.8	3.0	3.6	2.8	1.4	2.6

RESULTS.

Monthly Incidence of Zoster and Varicella.

In New York City during the 13 years, 1914–26, 95,994 cases of chicken-pox were reported. In Table I these cases have been set down

in a manner to indicate the incidence of the disease for each month during the 13 years. At the Bellevue clinic, 425 cases of herpes zoster were observed during the same period of 13 years, and 159



FIGS. 1–4. Curves showing the monthly incidence of varicella in New York City and herpes zoster in the Vanderbilt and Bellevue clinics.

cases were registered at the Vanderbilt clinic during the 5 years, 1922–26. In Tables II and III these cases of zoster have been arranged according to their monthly incidence. The significant features of the

information in Tables I to III have been graphically portrayed in Figs. 1 to 4.

Examination of the tables and figures dealing with the monthly incidence of varicella and zoster reveals the fact that a remarkably constant seasonal variation occurs in the number of cases of chicken-pox reported, while such variation in the prevalence of zoster is not very obvious. In fact, when the mean of a large number of cases over a period of many years is plotted (Fig. 4), the curve indicating the monthly incidence of zoster approaches a straight line.

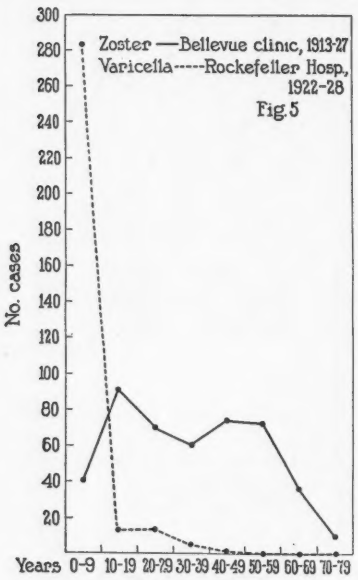


FIG. 5. Distribution curves illustrating the age incidence of varicella and herpes zoster.

Age Incidence of Zoster and Varicella.

In Table IV the age of 457 cases of zoster and of 318 cases of chicken-pox has been indicated. From the data in Table IV, distribution curves (Fig. 5) for the 2 diseases have been plotted. From the facts presented in Table IV and Fig. 5, it is quite evident that varicella occurs for the most part in individuals under 10 years of age, while zoster most frequently appears in the 2d, 3d, 4th, 5th and 6th decades of life. Obviously there is a striking difference in the age incidence of these diseases.

DISCUSSION.

The difference in the age incidence of herpes zoster and varicella has been well recognized for a long time (11) and facts concerning it are again presented in this paper merely for emphasis. That there is a constant seasonal variation in the prevalence of chicken-pox is also well known, and certain authors (9, 12) have recorded observations concerning the monthly incidence of zoster. Up to the present time,

however, very few attempts have been made to compare the number of cases of these diseases occurring simultaneously in the same locality. Perutz (13) made such a comparison in 2 districts of Vienna for the years 1923–25 and found that the curves of monthly incidence of the 2 diseases did not parallel each other. Moreover, Cantor (14) reports that on Christmas Island,[1] Straits Settlement, where excellent vital

TABLE IV.

A Tabulation of Varicella and Herpes Zoster Cases Showing the Difference in the Age Incidence of the 2 Diseases.

Herpes Zoster, Bellevue Clinic, 1913–27, 457 Cases.
Varicella, Rockefeller Hospital, 1922–28, 318 Cases.

Age	Zoster	Varicella	Age	Zoster	Varicella	Age	Zoster	Varicella	Age	Zoster	Varicella
0	1	18	19	13	2	38	8	1	57	6	0
1	1	32	20	7	5	39	3	0	58	8	0
2	1	27	21	6	0	40	12	1	59	6	0
3	4	48	22	6	1	41	7	0	60	9	0
4	2	38	23	6	1	42	10	0	61	2	0
5	6	45	24	4	0	43	5	0	62	4	0
6	6	39	25	8	4	44	6	0	63	0	0
7	7	17	26	13	0	45	7	0	64	5	0
8	7	11	27	10	3	46	8	0	65	6	0
9	6	8	28	4	0	47	5	0	66	4	0
10	11	4	29	7	1	48	5	0	67	1	0
11	11	2	30	4	0	49	10	0	68	3	0
12	9	2	31	5	0	50	12	0	69	3	0
13	13	1	32	9	1	51	6	0	70	1	0
14	10	2	33	5	2	52	11	0	71	0	0
15	3	2	34	7	0	53	7	0	72	2	0
16	9	0	35	8	0	54	5	0	73	2	0
17	6	0	36	6	0	55	5	0	74	2	0
18	6	0	37	5	0	56	6	0	75	3	0

statistics have been kept for 20 years, chicken-pox is unknown, while the usual amount of herpes zoster is observed. The statistical observations recorded in the present paper are further evidence in favor

[1] This island was uninhabited before 1888. The population, 500 to 1500, consists of Europeans, Indians, Malays, and Chinese. Both sexes and all ages are represented.

of the idea that the majority of cases of herpes zoster is not caused by the virus of varicella.

SUMMARY.

Varicella most frequently occurs in individuals under 10 years of age, while zoster as a rule is observed in persons beyond that age. The number of cases of varicella exhibits a markedly constant seasonal variation. The variations in the prevalence of herpes zoster are not regular and do not parallel those of varicella.

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RELATION OF VARICELLA TO HERPES ZOSTER.

II. Clinical and Experimental Observations.

By T. M. RIVERS, M.D., and L. A. ELDRIDGE, Jr., M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, February 13, 1929.)

In the preceding paper (1) the problem relating to the identity of the causal agents of varicella and herpes zoster was stated. The mere occurrence of varicella in an individual following exposure to herpes zoster might easily be explained upon coincidence. Certain other observations, however, dealing with complement fixation work, inoculation experiments, and protection tests conducted with convalescent sera deserve serious consideration.

Netter (2) and Netter and Urbain (3), de Lange (4), and others, because of their results with complement fixation tests, contend that the etiological agents of zoster and varicella are quite frequently identical. Lauda and Silberstern (5), however, were unable to confirm these observations. Kundratitz (6) and Lipschütz and Kundratitz (7) inoculated 28 children under 5 years of age with material from herpes zoster patients in the hope of immunizing them against chicken-pox. In 17 of the children a local reaction, characterized by papules and vesicles, was observed at the point of inoculation. The children were subsequently found to be immune to varicella. These writers also claim that injections of convalescent herpes zoster serum will protect exposed children against varicella. Lauda and Stöhr (8) inoculated 54 children with material from 17 cases of herpes zoster, and in none of them did they observe a local reaction at the site of the inoculation. Three of the inoculated children and 3 other uninoculated infants who came in contact with some of the zoster patients developed typical chicken-pox 2 or 3 weeks after exposure. These workers were unable to demonstrate that convalescent zoster serum protects against varicella and conclude from the results of their work that the majority of cases of zoster are not caused by the virus of chicken-pox.

Chicken-pox is a highly contagious disease, but for a long time doubt existed in regard to its inoculability. A great deal of this doubt arose from the fact that most of the experimental inoculations were made during epidemics of varicella and exposures to the disease under natural conditions could not absolutely be eliminated. Nevertheless, in spite of these difficulties, it has been shown (9) that

varicella is inoculable. Successful experiments, however, are not as constant as one might suppose when considering the extremely contagious nature of the disease. Evidence is accumulating in favor of the idea that zoster also is caused by a virus, but the question as to its inoculability in humans is still moot.

If it were possible without difficulty to produce varicella and zoster in animals, the relation of the two diseases would be settled quickly. It is very doubtful if the virus that causes the majority of cases of zoster has been propagated in experimental animals. With one exception this is also true in regard to varicella. Certain kinds of monkeys (*Cercopithecus sabæus* and *Cercopithecus lalandi*) are susceptible to the virus of chicken-pox. Even in these animals the results are irregular and the evidence of infection consists of the occurrence of acidophilic nuclear inclusions in affected cells of inoculated testicles. These inclusions, however, do not occur in controls and their appearance is regularly prevented by convalescent varicella serum (10, 11). In view of the regularity with which convalescent varicella serum prevents these inclusions in testicles inoculated with chicken-pox virus, it seemed likely that information regarding the identity of varicella and zoster might be obtained by conducting neutralization tests with varicella virus and convalescent zoster serum. It is with observations concerning these experiments that the present paper deals.

Methods and Materials.

Monkeys Employed.—Young male green monkeys (*Cercopithecus sabæus*) were used. Animals in which spermatogenesis had been established were discarded.

Neutralization Tests.—Emulsified papules and vesicles collected from varicella patients, usually within the first 72 hours of the disease, were used as virus. Non-immune serum was secured from chicken-pox patients during the first 72 hours of disease. Immune varicella serum was obtained from convalescent patients 14 to 22 days after the appearance of the rash. Immune zoster serum was collected from convalescent patients 23 to 28 days after the appearance of the eruption.

The papules and vesicles were excised under aseptic conditions and emulsified by grinding in a mortar moistened with Locke's solution. Sand was not used. The emulsified material was taken up in 0.5 to 2.0 cc. of Locke's solution and portions of it were mixed as desired with equal amounts of non-immune serum, immune varicella serum, or immune herpes zoster serum. Measured amounts (0.25 cc.) of the mixtures were then injected into the testicles of monkeys. More than 45 minutes never elapsed between the collection of the varicella virus and its injection into the animals.

Removal and Examination of Testicles.—It was previously shown (10, 11) that nuclear inclusions are present in the testicles of green monkeys on the 5th and 6th days after inoculation with chicken-pox. Consequently, in the experiments reported at the present time, the monkeys were castrated[1] on the 5th or 6th day following inoculation. The testicles removed for histological studies were fixed in Zenker's fluid, sectioned, and stained with eosin and methylene blue. A careful search for eosin-staining nuclear inclusions was made in numerous sections of each testicle. Details concerning the tinctorial reactions of the inclusions are given by Tyzzer (12), Lipschütz (13), and Goodpasture (14).

Results.—Testicles in which nuclear inclusions were found were considered infected with varicella virus. If no inclusions were found, infection was considered not to have occurred. In this type of work, positive results are naturally much more significant than are negative ones.

EXPERIMENTAL.

In the study of the relation of varicella to herpes zoster 4 neutralization experiments were performed.[2] A detailed account of each follows.

Experiment I.

The first experiment was conducted in order to determine whether the serum of an adult, who had had chicken-pox in childhood, would neutralize varicella virus.

Serum was collected from J., 35 years of age, who had chicken-pox when 5 years old. Non-immune serum was obtained from W. R. during the first 48 hours after the appearance of the varicella rash. Virus in the form of 4 varicella vesicles was secured from B. The serum to be tested, from J., was mixed with an equal amount of virus, and then 0.25 cc. of the mixture was injected into each testicle of Monkey 1. Non-immune serum, W. R., was also mixed with virus, and 0.25 cc. of the mixture was injected into each testicle of Monkey 2. 5 days later the testicles were removed and examined in the usual manner for the presence of acidophilic nuclear inclusions. They were found in the testicles of both monkeys.

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[1] All operative procedures were conducted under anesthesia.

[2] Because of the scarcity of green monkeys, control tests with convalescent varicella serum were at times omitted. In fact, it is not essential to use this control with each experiment, inasmuch as convalescent varicella serum has never failed to prevent the appearance of inclusions in testicles inoculated with varicella virus (11).

The results of the above experiment indicated to us that the serum from an individual who had had varicella many years previously did not contain sufficient antibodies to prevent the appearance of inclusions in testicles inoculated with chicken-pox virus. Consequently it was deemed safe in this work to use convalescent zoster serum obtained from individuals who a number of years earlier in life had passed through an attack of varicella.

Experiment II.

In this experiment an attempt was made to ascertain whether the serum from a case of so called idiopathic herpes zoster was capable of neutralizing varicella virus.

C. S., male, white, 16 years old. No history of having had chicken-pox or zoster previously. No exposure recently to either disease. Patient seen on the first day of the zoster eruption which was distributed over the right sacral and iliac regions; serum collected. Lesions healed slowly. Serum again collected 23 days after onset of the disease.

In the neutralization tests, 4 monkeys were used. Virus, 9 lesions, was collected from 3 varicella patients (G. K., G. M., H. B.). Non-immune serum (W. R.), immune serum (M. W.), and serum collected from C. S. on the 1st and 23d days after the appearance of the zoster eruption were employed. Virus and non-immune serum were injected into Monkey 3; virus and convalescent varicella serum into Monkey 4; virus and early zoster serum into Monkey 5; virus and convalescent zoster serum into Monkey 6. 6 days after the inoculations, the testicles were removed, fixed, sectioned, and stained. Upon examination no inclusions were found in any of the sections.

In view of the negative results in all the monkeys, the experiment was repeated. At this time, however, only non-immune serum (W. R.) and convalescent zoster serum (C. S.) were used. The virus, 6 varicella vesicles, was obtained from A. M. and W. McK. Virus and non-immune serum was injected into Monkey 7; virus and convalescent zoster serum into Monkey 8. 5 days later the testicles were removed and examined in the usual manner. In sections from the testicles of Monkey 7 numerous nuclear inclusions were found, while none were seen in those from Monkey 8.

From the results of this experiment one might conclude that the convalescent zoster serum neutralized the varicella virus. Monkeys, however, display a certain amount of irregularity in their susceptibility to chicken-pox virus. Consequently one set of negative findings is not highly significant.

Experiment III.

The following test was made to determine whether the serum from a patient who developed herpes zoster following the administration of salvarsan would neutralize chicken-pox virus.

M. R., female, colored, age 36. No history of previous attacks of chicken-pox or herpes zoster. No known recent exposure to either disease. ++++ Wassermann. On Jan. 13, 1927, at the Vanderbilt clinic, the patient received her first intravenous injection of salvarsan. 24 hours later a burning sensation was felt over the left shoulder, and 48 hours following the treatment blisters were seen over the left side of neck and left shoulder. Patient was seen at the Rockefeller Hospital for the first time on Jan. 21 and was found to have typical herpes zoster. The lesions healed slowly. Convalescent serum was collected on Feb. 11.

Virus, 4 fresh vesicles, was collected from a varicella patient on the 3d day of eruption. Virus and non-immune serum (W. R.) were injected into Monkey 9; convalescent zoster serum (M. R.) and virus were inoculated into Monkey 10. 5 days after the injections the testicles were removed and examined for the presence of acidophilic nuclear inclusion. In the testicles of both animals many were found.

In this experiment it seems that the convalescent zoster serum did not neutralize the chicken-pox virus.

Experiment IV.

The experiment described below is the most important one in the series. In it was used serum from a convalescent zoster patient who had had varicella $7\frac{1}{2}$ years previously and from whom a sister appeared to have contracted chicken-pox through exposure to her during the attack of zoster.

M. B., female, white, age 8. No history of previous attacks of zoster. Had chicken-pox when 6 months old. No recent exposure to varicella or zoster. Attends a public school. On Oct. 24, 1926, the patient developed blisters over the right side of the body. She was not quarantined and came in contact with relatives, classmates, and playmates. On Nov. 1, she was seen at the Vanderbilt clinic and referred to the Rockefeller Hospital. At that time the patient presented the picture of typical herpes zoster. The eruption was on the right side of the body over an area supplied by the 9th and 10th thoracic nerves. The child was not admitted to the hospital, but was seen from time to time in the clinic. Dr. E. visited the home on several occasions and a nurse observed the children in the patient's class at school. The lesions on the child healed slowly, and convalescent serum was obtained on Nov. 20, 26 days after the appearance of the eruption.

V. B., age 3, a sister who had never had varicella or zoster was constantly exposed to the patient. On Nov. 13, 19 days after the appearance of zoster in M. B., a chicken-pox eruption was observed on V. B. The child was admitted to the Rockefeller Hospital and after passing through a typical attack of varicella was discharged on Nov. 27. Just prior to discharge convalescent serum was obtained.

The parents said that V. B. had not been exposed to varicella, yet she developed the disease 19 days after exposure to herpes zoster in her sister. This, then, is an excellent example of chicken-pox developing in a child after exposure to herpes zoster. Many such examples have been reported and have been adduced as evidence of the identity of the two diseases. Upon pursuing the matter further, however, it was found that M. B.'s class at school consisted of 33 children, 21 of whom had never had varicella. All of them were exposed to M. B., yet not a case of chicken-pox developed as a result of the exposure.

Four monkeys were used in the neutralization tests. The virus consisted of 12 vesicles removed from 3 varicella patients (J. R., W. R., V. D.). Non-immune serum (W. R.), chicken-pox convalescent serum (M. W.), convalescent serum from M. B., and convalescent serum from V. B. were respectively mixed with equal amounts of virus. 0.25 cc. of the mixtures were then injected into the testicles of monkeys; virus and non-immune serum into Monkey 11, virus and convalescent varicella serum into Monkey 12, virus and convalescent serum from M. B. into Monkey 13, virus and convalescent serum from V. B. into Monkey 14. 5 days after the injections the testicles were removed and examined in the usual way for the presence of nuclear inclusions. Sections from Monkeys 11 and 13 showed numerous inclusions, while those from Monkeys 12 and 14 revealed none.

The results of the experiment indicate that the non-immune serum and the serum from the child (M. B.) convalescent after zoster did not neutralize varicella virus, while convalescent varicella serum of extraneous derivation and convalescent serum from the sister (V. B.) who had just had chicken-pox did inhibit its action. In view of these findings, together with the failure of varicella to develop in any of the classmates of the child (M. B.) who had zoster, one is justified in concluding that the relation of chicken-pox to zoster in the two sisters, V. B. and M. B., was apparent rather than real.

A summary of the experiments dealing with the neutralization of varicella virus by convalescent herpes zoster serum is given in Table I. An analysis of the results reveals that, under the conditions of the tests, sera from individuals who had had varicella $7\frac{1}{2}$ to 30 years previously did not neutralize varicella virus. This fact does not imply that these individuals were susceptible to chicken-pox and that their sera did not possess a certain amount of neutralizing power for varicella

virus. The experiments also indicate that 2 of 3 convalescent herpes zoster sera did not neutralize varicella virus while in each of 2 in-

TABLE I.

Summary of Neutralization Experiments.

Experiment	Monkey	Inoculum	Results
1	1	Varicella virus + non-immune serum	+
	2	" " + serum from adult who had varicella 30 years previously	+
2	3	" " + non-immune serum	−
	4	" " + convalescent varicella serum	−
	5	" " + serum from patient early in course of herpes zoster	−
	6	" " + serum from patient convalescent from herpes zoster	−
2	7	" " + non-immune serum	+
Repeat	8	" " + convalescent herpes zoster serum	−
3	9	" " + non-immune serum	+
	10	" " + convalescent herpes zoster serum	+
4	11	" " + non-immune serum	+
	12	" " + convalescent varicella serum	−
	13	" " + convalescent herpes zoster serum from an individual (M. B.) who had had varicella 7½ years previously	+
	14	" " + convalescent varicella serum (V. B.)	−

Monkeys inoculated in testicles.

+ indicates infection by virus, determined by presence of nuclear inclusions in sections of inoculated testicles.

− indicates no infection by virus, determined by absence of nuclear inclusions in sections of inoculated testicles.

stances[3] convalescent chicken-pox serum did neutralize it. An adequate explanation of why varicella virus appeared to be neutralized by one of the convalescent zoster sera is lacking. It is certainly not

[3] Convalescent varicella serum regularly prevents the occurrence of nuclear inclusions in monkeys' testicles inoculated with varicella virus (11).

safe to conclude, even though the experiment was repeated, that the herpes zoster in that case was caused by varicella virus. From the experiments reported in this paper and those previously recorded in regard to varicella in monkeys (10, 11), one is justified in concluding that herpes zoster is not always produced by varicella virus. Such is the case even in instances where clinical observations might lead one to suspect that the causal agents of the two diseases are identical.

DISCUSSION.

Many investigators believe that certain cases of zoster are caused by a specific virus. Instances, however, of "recurring zoster," of zoster following mechanical or drug injuries to nerves, and of zoster occurring in patients with tabes, tuberculosis of the spine, and cord tumors have led numerous observers to consider that this type of the disease is due not to an infectious agent, but to trophic changes in the skin incident to nerve injury. Regardless of the conditions under which herpes zoster occurs, the character of the lesions has induced a number of workers familiar with virus diseases to entertain the idea that it is caused by some kind of virus.

The question as to whether zoster is caused by one or by several viruses naturally arises. The pathological changes observed in zoster lesions are strikingly similar to those seen in herpes simplex and varicella. In fact, lesions from these three diseases cannot be differentially diagnosed one from another by means of the microscope. As a group, however, they can be separated from other skin lesions occurring in man. This is made possible by the occurrence of acidophilic bodies in the nuclei of affected cells. From records of work dealing with the identity of herpes simplex and herpes zoster (15) it seems unlikely that the etiological agents concerned in the majority of the cases of these two diseases are identical.

It now remains to consider the relation of zoster to chicken-pox. The studies presented in the preceding paper (1), the experiments recorded in the present one, and the fact that an attack of herpes zoster as a rule does not protect against varicella and *vice versa* (16, 17, 18, 22) seem to indicate that the majority of the cases of the two diseases is not caused by the same virus. There is no good reason, however, why one should say that the virus of chicken-pox cannot under

certain conditions produce localized lesions clinically indistinguishable from herpes zoster. It probably does, but only rarely.

The conditions under which the virus of varicella causes a disease clinically similar to herpes zoster are probably dependent upon the rôle injury or irritation plays in the localization of many viruses. The viruses of measles (19) and varicella (20) localize in areas of irritated skin as is evidenced by a marked increase in the number of lesions appearing in such areas. Therefore, in hospitals where large numbers of tuberculous children and syphilic infants undergoing treatment with arsenicals are cared for, a few cases of clinical zoster may occur during an epidemic of varicella. In all probability, under these conditions, some if not all of the so called zoster is caused by chickenpox virus. Consequently it would be better to diagnose such cases as varicella with an abnormal localization of the rash.

It is not likely that trophic changes incident to nerve injury alone or to drug poisoning alone, *e.g.*, with arsenic, are capable of producing herpes zoster. If such were the case, the disease should be seen more often under these conditions. The fact that viruses tend to localize in irritated tissues may also account for the relation that has been observed to exist between certain cases of herpes zoster and nerve injury due to chemical, physical, or disease-producing agents. Whether the virus localizes primarily in ganglia and subsequently travels by way of nerves to the skin or whether the localization is only in the skin, the site being determined by circulatory and other changes incident to the injury of nerves supplying the region (21), is not definitely known. There is no reason why the primary localization of the virus may not occur in either place. The presence or absence of pain in zoster may in some instances be dependent upon the place at which the virus localizes.

Since in the majority of cases of herpes zoster the disease is caused neither by the virus of herpes simplex nor by the virus of varicella, is there any evidence at present that a special virus is concerned in its production? There is no direct proof that such a virus exists, yet the indirect evidence is suggestive. In the first place, the pathological picture presented by zoster lesions is one that has not been shown to occur in the absence of some kind of virus. Although zoster is not, as a rule, highly contagious, it breeds true when occur-

ring epidemically, *i.e.*, zoster gives rise to zoster (15, 22). Finally, in the majority of instances one attack of zoster confers an immunity against a second attack of the disease but not against an infection with herpes simplex and varicella viruses.

SUMMARY.

Experiments and clinical observations dealing with the identity of the viruses of varicella and herpes zoster were presented. The results indicate that the etiological agents concerned with these two diseases are in the majority of instances not identical.

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IMMUNOLOGIC REACTIONS WITH TOBACCO MOSAIC VIRUS.

By HELEN A. PURDY.

(From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, and the Boyce Thompson Institute for Plant Research, Inc., Yonkers.)

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Ever since Mayer (1886) demonstrated the infectious nature of tobacco mosaic disease, investigations have been carried out to determine the true nature of the etiologic agent of this malady. Many of the objections to the parasitic theory based on the resistance of the infective agent to chemicals can be met with the argument that the usual methods for the determination of these reactions in the case of known microorganisms are crude when applied to the study of filterable viruses (Ford, 1927). Studies on the purification of virus have been made which may overcome some of the difficulties (McKinney 1927; Vinson and Petre 1929; and Brewer, Kraybill and Gardner 1929). The apparently high resistance to heat exhibited by virus is no greater than that shown by bacterial spores (Duggar and Armstrong 1923). Spores of *B. subtilis* show an increased resistance to heat when tested in vegetable infusions (Williams 1928).

The properties of tobacco virus are frequently likened to those of enzymes. An apparent similarity between them may be due to the fact that they have both been studied in tissue extracts and in an impure state. Falk (1924) maintains that the properties attributed to enzymes are in reality chiefly those of the medium in which they are tested. Allard remarked fourteen years ago that: "It may at least be said that the theory of a parasitic origin for the disease more consistently accounts for all the facts at hand than any enzymic conception yet evolved. It seems not only needless but illogical to abandon a simple, direct explanation for one which leads to complexity of thought and yet fails to correlate all the facts at hand." (Allard 1915).

Various investigators have proposed different hypotheses to explain the origin of tobacco mosaic disease. Some of them express the opinion that the malady is due to purely physiologic response to unfavorable environmental conditions (Egiz 1912; Hunger 1902, 1905 a, b; and Sturgis 1899). Hunger maintains that a phytotoxin is generated spontaneously in the plant which upon transmission to normal plants proves capable of stimulating them also to the production of the phytotoxin. Beijerinck attributes the disease to a "contagium vivum fluidum" (1898), while other workers think the excessive accumulation of oxidizing enzymes is responsible for the morbid condition of the plant (Heintzel 1900, Woods 1899,

1902, and Chapman 1913). Iwanowski, although unable to cultivate a microorganism from affected plants, considers the disease as bacterial in origin (1903). Allard (1916) expresses the opinion that the causal agent is an ultramicroscopic parasite. Several investigators have described microorganisms which they believe to be closely associated with the disease (Palm 1922; Jones 1926 a, b; Link, Jones, and Taliaferro 1926; Jones 1928; and Eckerson 1926). One of these supporters of the parasitic theory of tobacco mosaic disease claims that the foreign cell inclusions, characteristically present in tobacco mosaic tissue are the etiologic factor (Palm 1922).

Olitsky's claim of having cultivated the infective agent of tobacco mosaic disease[1] *in vitro* in the absence of living host cells (1924) is as yet uncorroborated (Mulvania 1925; Purdy 1926; Goldsworthy 1926; and Smith 1928).

Mulvania (1926) injected from 1 to 3 cc. of virus-sap in one ear of a rabbit and 20 minutes later tried to recover the virus by drawing a sample of blood from the uninoculated ear. The virus was not recoverable. He also showed that when normal, fresh, rabbit blood is mixed with virus extract and allowed to stand for 24 hours, the infectivity of the virus is not destroyed but under the same conditions, a filtrate of virus extract is rendered non-infectious. No attempt to produce an antiserum to virus-sap was reported by Mulvania.

Until the recent work of Dvorak (1927), on a virus of potato mosaic disease, no one had undertaken an investigation of the antigenic properties of virus-sap. Dvorak produced antisera in rabbits to sap from healthy potato plants and sap from potato affected with mosaic disease.[2] Precipitin reactions showed that the two antisera had precipitins in common, but that they both exhibited a higher titer for the homologous than for the heterologous antigen. The explanation offered for these results is that the virus had in some way altered the antigenic property of the globulins present in the healthy potato plant.

A study of the antigenic properties of virus-sap of tobacco mosaic should throw light upon the true nature of the virus. Therefore, the experiments described in this paper, reported previously in preliminary form (Purdy 1928), were undertaken.

Method.

Preparation of Antigens.—As a source of antigens young tobacco plants of a Turkish variety were selected because of a low nicotine-content, which would minimize any toxic effect the alkaloid might have on the rabbits used for immunization. Leaves were collected from healthy tobacco plants and from plants

[1] The host plant in this case was tomato.

[2] Although the filterability of the virus of potato mosaic disease has never been proved, it is nevertheless regarded as belonging to the class of filterable viruses.

that had been infected with mosaic disease for from 10 days to 6 weeks. The particular tobacco virus used was that of Johnson's common field mosaic disease.[3] The diseased and healthy leaves were kept in two separate lots and prepared in vessels, sterilized each time after use to avoid any chance of contaminating the normal sap with virus-sap. Three medium-sized leaves were ground to a pulp in a mortar with 8.5 cc. of saline solution (0.85 per cent). The extract was then centrifugalized for about 5 minutes at a moderate speed and the supernatant fluid was used for injection. At first, the antigens were preserved with 0.5 per cent carbolic acid solution and stored in an ice-chest during the intervals between injections, but later fresh antigen was prepared for each injection to eliminate

TABLE I.

Hyperimmunization of Rabbits.

Rabbit No.	Antigen	No. of injections	Total amount of antigen injected	Period of hyper-immunization	No. of days after last injection, rabbit was bled
1	N*	6	23.0	Feb. 10–Mar. 6	6
2	N	5	16.5	Apr. 10–Apr. 23	10
3	N	6	19.0	Apr. 10–May 11	0†
4	V*	6	21.0	Feb. 10–Mar. 6	6
5	V	5	16.5	Apr. 10–Apr. 23	10
6	V	13	22.8	Apr. 10–June 7	14
7	V	7	9.2	July 25–Aug. 22	0†

* N = Normal sap.
V = Virus-sap.
† Rabbit died before bleeding.

the possibility of the proteins in the extracted sap becoming denatured upon standing.

Immunization of Rabbits.—Each rabbit received from 5 to 13 injections of antigen in the marginal ear-vein, the amount injected at one time varying from 0.5 to 5 cc. Since the rabbits showed toxic reactions when as much as 5 cc. were injected at one time, it was not deemed advisable to use a larger amount of sap extract. The usual interval between injections was 3 to 4 days. Before the fifth and subsequent injections, the rabbit was desensitized intravenously first with 0.5 cc. of antigen, otherwise severe anaphylactic symptoms were evinced. Four rabbits were injected with virus-sap and 3 with normal sap. Table I contains more details of the procedure followed in the injection of individual rabbits.

[3] The original source of the virus was mosaic material supplied by Doctor James Johnson several years ago and subsequently kept free from contamination by other strains of virus at the Boyce Thompson Institute.

Collection of Serum.—In general, samples of serum were drawn from an ear-vein for control purposes, prior to the first injection of the antigen. Thereafter, frequent samples of serum were taken from an ear-vein during the course of injections for use in various tests. From 6 to 14 days after the last injection, the animals were etherized and bled to death aseptically from the carotid artery. The antiserum was allowed to separate from the clot overnight and was pipetted off the following morning, inactivated for one-half hour at 56°C. (water-bath) and stored in sterile tubes in an ice-chest.

Testing of Serum for Antibodies.

Preliminary alexin-fixation experiments were carried out in order to determine whether or not antibodies to virus and normal sap had been produced in the injected rabbits. It was found that in the presence of the homologous antigen, a given amount of serum from the injected animals would fix alexin completely, whereas the same amount of antigen and normal rabbit serum gave no fixation, that is, complete hemolysis occurred on adding sensitized sheep cells. The usual control tubes for antigen, hemolytic system, serum, and cells were included in the tests. All showed that the experiments were properly controlled.

The findings of these preliminary tests indicated that antibodies to virus and normal sap had been produced in the injected rabbits. Alexin-fixation experiments were then planned for the titration of both antigens with antisera to virus-sap and antisera to normal sap in order to ascertain whether or not the antisera contained alexin-fixing antibodies in common.

Alexin-Fixation Experiments.

The antisera and normal sera were heated for one-half hour at 56°C. (water-bath) just prior to use. Constant amounts (0.05 cc.) of undiluted serum were placed in Wassermann tubes. Fresh guinea-pig serum was then added of a dilution that would contain 2 units of alexin in each 0.1 cc. The antigens were prepared as for injection in the rabbits and were diluted further with saline solution (0.85 per cent). Decreasing amounts of antigen-dilution were pipetted into a series of tubes. The volume of liquid in each tube was then brought up to 0.25 cc. with saline solution. The entire mixture was shaken and placed in a water-bath for one hour at 37°C. At the end of the hour of incubation, sheep cells, previously sensitized with rabbit sensitizer, were added in 0.2 cc. amounts and the tubes were returned to the water-bath for 15 minutes. In some of the experiments, the readings were made immediately after removal from the water-bath; in other experiments the readings were made after the tubes had stood in the ice-chest overnight and the cells had settled out by gravitation. In general, it was found that a satisfactory and quicker method of obtaining the readings was to cool the

tubes under running water immediately after removal from the water-bath, and then centrifugalize them at a low speed for a few moments to throw down any non-hemolyzed cells present. In most of the tests, Madsen's scale was employed for determining the percentage hemolysis occurring in any of the tubes. Using the contents of tubes showing complete hemolysis as the stock solution, dilutions with saline solution were made showing 10 to 90 per cent hemolysis, by increments of 10 per cent.

Three separate experiments were carried out in which 3 different antivirus and 3 different antinormal sera were tested individually for their ability to bind alexin in the presence of virus and normal sap. The results of these experiments showed the smallest amount of antigen used that would react with 0.05 cc. of undiluted antiserum to fix a given amount of alexin. For the sake of discussion, these minimal amounts wherever available have been brought together in Table II. The amount of antigen used is expressed in the equivalent of undiluted sap to facilitate a comparison of the results.

Analysis of the Findings.—In all of 3 experiments (Table II) both antisera fix alexin in the presence of either antigen.

In all 5 titrations (Table II) antivirus sera show a higher titer for the homologous than for the heterologous antigen.

In all 3 experiments (Table II) antivirus sera possess a higher titer for virus than the antinormal sera for the same antigen.

In 3 out of 5 titrations (Table II, Experiments I, II, and IIIa) the antinormal sera exhibit a higher titer for the homologous antigen than for the heterologous antigen. Complete results were not obtained in the other 2 titrations (Table II, Experiment IIIb and c).

In 2 out of 5 titrations (Table II, Experiments I, and IIIa) the antinormal sera have a higher titer for normal sap than the corresponding antivirus sera for the same antigen; in one instance the titer of the antinormal and antivirus sera for normal sap is the same (Table II, Experiment II); in the other 2 titrations, the incomplete results would indicate that the antinormal sera have a lower titer for normal sap than the corresponding antivirus sera (Table II, Experiment IIIb, c).

Discussion.—In interpreting the results of the alexin-fixation experiments summarized in Table II, two possibilities should be taken into consideration—the differences that may exist in both the titer of the

antisera and the concentration of the antigens. These two factors influence the results, but if properly accounted for, they do not invali-

TABLE II.

Minimal Amounts of Antigen Required for Alexin-fixation.

Experiment No.	Undiluted serum (56°C.)	Undiluted antigen	Results	
			Hemolysis	Fixation
	cc.	*cc.*		
I	AV† 4	.006 V	—	Complete
	AV 4	.02 N*	Partial	—
	AN 1	.02 V*	Partial	—
	AN 1	.002 N	—	Complete
II	AV 4	.0015 V	—	Complete
	AV 4	.008 N	—	Complete
	AN 1	.01 V	—	Complete
	AN 1	.008 N	—	Complete
IIIa	AV 4	.001 V	—	Complete
	AV 4	.03 N	—	Complete
	AN 1	.03 V	—	Complete
	AN 1	.02 N	—	Complete
IIIb	AV 6	.0006 V	—	Complete
	AV 6	.1 N	—	Complete
	AN 3	.05 V	—	Complete
	AN 3	.02 N*	75%	—
IIIc	AV 5	.0006 V	—	Complete
	AV 5	.05 N	—	Complete
	AN 2	.1 V	—	Complete
	AN 2	.02 N*	85%	—

* Minimal amount of antigen was not determined.

† AV = Antiserum to virus-sap.
AN = Antiserum to normal sap.
V = Virus-sap.
N = Normal sap.

date the conclusions that may be drawn from these results. The following discussion will demonstrate this point.

Since antivirus serum fixes alexin in the presence of normal sap, it is fair to assume that antigenic substances in the normal sap were also

present in virus-sap used for the production of the antivirus serum. Likewise, since antinormal serum binds alexin in the presence of virus-sap, the reaction may be attributed to antigenic substances in virus-sap that were also present in normal sap. Without further knowledge of the antigenic nature of normal and virus-sap, the results obtained from these experiments may be examined to advantage on the assumption that the two antigens, virus and normal sap, are identical in composition. It is then possible to compare the titer of the antisera and the concentration of the antigens from the results of a single experiment in which antivirus and antinormal sera are titrated with the same preparations of homologous and heterologous antigens. The normal antigen and antinormal serum should be used as the basis for comparing the titer of the antisera and the concentration of the antigens, since the assumption that virus-sap is identical to normal sap in composition remains to be proved.

For example, in Experiment I (Table II) comparing the reaction of the 2 antivirus and antinormal sera to normal sap, the antinormal serum is the higher titered one. By further comparison of the reactions of antinormal serum with virus and normal sap, the former antigen appears to be less than one-tenth the concentration of the latter. By a similar comparison of the titrations in Experiment II (Table II), both antisera have the same titer, but virus-sap appears to be a somewhat weaker antigen than normal sap.

In all 5 sets of titrations, antivirus sera exhibit a higher titer for the homologous than for the heterologous antigen. On the other hand, the incomplete results of Experiment IIIb (Table II) indicate that the antinormal serum may manifest a higher titer for the heterologous than for the homologous antigen, depending in this instance upon the concentration of the antigens. Also, the antinormal sera may show a lower titer for the homologous antigen than that of the corresponding antivirus sera (Table II, Experiment IIIb, c) because of the higher titer of the latter. These results demonstrate the importance of titrating the 2 antisera with both antigens. Without the information thereby gained relative to the concentration of antigen and titer of antiserum, no dependable conclusions can be drawn from the results of a titration.

A careful study of the results obtained from these experiments makes

it evident that differences in the titer of the antisera or concentration of the antigens, identical in composition, are sufficient to explain the reactions of antinormal sera with both antigens, but not to account for the reactions of antivirus sera with virus and normal sap. For instance, in Experiment I (Table II) the antivirus serum is weaker in titer for normal sap than the corresponding antinormal serum, and the normal sap seems to be more highly concentrated than the virus-sap, from a comparison of the reactions of antinormal serum with both antigens. Under these conditions and with antigens of identical composition, the antivirus serum would be expected to show a higher titer for normal than for virus-sap, but in reality the reverse is true and antivirus serum exhibits a higher titer for virus than for normal sap. The only adequate explanation for such a result is that the 2 antigens differ in composition. Virus-sap apparently contains some antigenic substance or substances either not found in normal sap, or only in small amounts. It is possible that some foreign antigenic substance is present in virus-sap not found in normal sap, or that some of the normal antigens present in healthy tobacco sap have been altered by mosaic disease, thereby changing their antigenic properties.

The 2 antisera were now tested for the presence of precipitins.

Precipitin Experiments.

Four precipitin tests were set up, using equal parts of undiluted antiserum and antigen-dilution, varying from 1:10 to 1:100,000. The antigens were prepared as for injection in rabbits but it was found necessary to centrifugalize the extracts at high speed for 15 minutes to obtain sufficiently clear antigens. The supernatant fluid was further diluted with saline solution (0.85 per cent). The serum was first pipetted into precipitin tubes and the antigen-dilution was slowly "layered" on top of the serum. After incubation for 1 hour at 37°C. (water-bath), the tubes were examined for the presence of a ring of precipitate, formed at the region of contact between serum and antigen. The contents of the tubes were then thoroughly shaken and placed in an ice-chest overnight. On the following morning a record was made of the precipitate formed in the tubes.

Analysis of the Findings.—No precipitate was formed in a dilution of antigen exceeding 1:100.

A precipitate formed in the case of both antisera in the presence of either antigen.

A very slight precipitate formed in a mixture of normal sap and normal serum when the antigen was undiluted.

The virus-sap produced a heavier, more flocculent precipitate than the normal sap with both antinormal and antivirus serum. A greater amount of precipitate was obtained in a mixture of antivirus serum and virus-sap than in the case of antinormal serum and virus-sap.

Discussion.—The fact that the antigens failed to give a precipitate in a dilution exceeding 1:100, may be due to the fact that the antigen is prepared by the extraction of whole sap high in water-content. The antigens referred to as "undiluted" are in reality considerably diluted.

The heavier precipitate characteristically formed in a mixture of antivirus serum and virus-sap strongly suggests the presence of a specific precipitin for virus-sap in the antivirus serum. In comparing the reactions of the 2 antisera with their homologous and heterologous antigens, the same objections raised to a comparison of the results in the alexin-fixation experiments should be taken into consideration. In these tests, also, the titer of the 2 antisera and the concentration of the antigens may vary. It was concluded, therefore, in the face of these difficulties that the presence of specific precipitin for virus-sap in antivirus serum could be more readily demonstrated by precipitin-absorption experiments.

Precipitin-Absorption Tests.

After a number of preliminary tests, it was found that the most satisfactory method for the absorption of the precipitins from the serum was as follows:

The antigens were freshly prepared as for the precipitin experiments and used in a dilution of 1 part of extracted sap to 4 parts of saline solution. The antisera were absorbed by adding 1 cc. of antigen-dilution to 2.5 cc. of inactivated serum and incubating for 1 hour at 37°C. (water-bath). The precipitate formed was then removed by centrifugalization and 1 cc. of antigen-dilution was added to the clear supernatant fluid. This process was repeated until none of the tubes showed any precipitate upon removal from the water-bath. The tubes were then set in the ice-chest overnight to allow ample opportunity for a precipitate to form. If the tubes contained no precipitate on the following morning the absorption of the serum was considered complete. The absorbed serum was then tested with both antigens for the presence of precipitins, as described in the precipitin experiments. During the process of absorption, several precipitin tests were carried out with the partially absorbed sera.

Two different antivirus sera and 1 antinormal serum were used in the 3 absorption tests. Two portions each of antivirus and antinormal serum were absorbed separately with both normal and virus-sap. Control tubes of antisera were included, treated in a similar manner to the sera absorbed except that saline was added each time in the place of antigen. Other appropriate control tubes were also used.

Analysis of the Findings.—All of the precipitins to normal sap in both antisera were completely absorbed by either antigen.

After complete absorption of antivirus serum with normal sap, a heavy precipitate was formed upon the subsequent addition of virus-sap.

Discussion.—During the process of precipitin-absorption, the antinormal serum was usually absorbed completely by the first addition of antigen while the largest amount of antigen used in these tests was required by the antivirus serum for complete absorption with virus-sap. For uniformity of method, the same quantity of antigen was used in every case for the absorption of the 4 different lots of serum in a single experiment. Consequently, it was necessary to set up complete precipitin tests at various stages during the process of absorption, otherwise the results would be incomplete, for the final precipitin tests showed that some of the absorbed sera were so highly diluted that no reaction occurred in the corresponding control tubes of unabsorbed serum.

The point of most interest in these experiments has been fully demonstrated by this method of precipitin-absorption, namely, that after complete absorption of an antivirus serum with normal sap, the absorbed serum will still give a heavy precipitate with virus-sap. As stated in the discussion of the results of the alexin-fixation experiments, the specific reaction may be due either to a foreign antigenic substance present in the virus-sap, or to the formation during the course of mosaic disease of an altered plant protein.

As a logical sequence to the precipitin-absorption tests, it was decided to repeat the experiments and instead of using tobacco sap for the final precipitin tests to substitute sap from healthy and mosaic diseased hosts of the virus other than tobacco.

Precipitin Tests with Tobacco Virus from Other Hosts.—Tomato, pepper, and petunia plants, common hosts of tobacco mosaic virus, were inoculated with the

TABLE III.

Precipitin-absorption Tests with Virus from Various Hosts of Tobacco Mosaic Virus.

Tube No.	Serum	Antigen used for absorption	Undiluted absorbed serum	Antigen dilution 1:10	Results	
					After incubation for 1 hr. at 37°C. (water bath)	After standing in ice-chest overnight*
			cc.	*cc.*		
1	AV†	N tobacco	.2	.2N tobacco	No ring	0
2	"	" "	.2	.2V tobacco	Ring	+++
3	"	" "	.2	.2N tomato	No ring	0
4	"	" "	.2	.2V tomato	Ring	+++
5	"	" "	.2	.2N pepper	No ring	0
6	"	" "	.2	.2V pepper	Heavy ring	++++
7	"	" "	.2	.2N petunia	No ring	0
8	"	" "	.2	.2V petunia	Very faint ring	++
9	"	" "	.2	.2 saline	No ring	0
10	"	" "	.2 saline	.2N tobacco	" "	0
11	"	" "	" "	.2V tobacco	" "	0
12	"	" "	" "	.2N tomato	" "	0
13	"	" "	" "	.2V tomato	" "	0
14	"	" "	" "	.2N pepper	" "	0
15	"	" "	" "	.2V pepper	" "	0
16	"	" "	" "	.2N petunia	" "	0
17	"	" "	" "	.2V petunia	" "	0
18	"	Saline	Unabsorbed serum .2	.2N tobacco	No ring	++
19	"	"	"	.2N tomato	Ring	+
20	"	"	"	.2N pepper	No ring	+
21	"	"	"	.2N petunia	" "	±
22	"	"	"	.2V tobacco	" "	++
23	"	"	"	.2V tomato	Heavy ring	+++
24	"	"	"	.2V pepper	Ring	++++
25	"	"	"	.2V petunia	Ring	++
26	"	"	"	.2 saline	No ring	0

* The following symbols are used:

0 = no precipitate.
± = very slight precipitate.
+ = slight precipitate.
++ = moderate precipitate.
+++ = heavy precipitate.
++++ = very heavy precipitate.

† AV = antiserum to virus-sap.
AN = antiserum to normal sap.
V = virus-sap.
N = normal sap.

strain of virus used throughout these experiments. The identical procedure used above in the precipitin-absorption tests was followed. Four sets of experiments were carried out. The results obtained from one of these tests are recorded in Table III. A parallel series of tubes with the corresponding unabsorbed serum was included in these experiments.

Analysis of the Findings.—When antiserum to virus-sap of tobacco was completely absorbed with normal tobacco sap (Table III):

(*a*) No precipitate was formed upon adding normal sap of tobacco, tomato, pepper, or petunia plants.

(*b*) A heavy precipitate was formed upon the addition of virus-sap of tobacco, tomato, pepper, and petunia plants.

Unabsorbed antiserum to virus-sap of tobacco produced some precipitate when mixed with the normal sap of tobacco, tomato, pepper, and petunia plants.

Discussion.—Since the 4 different host plants, tobacco, tomato, pepper, and petunia used in these experiments are all members of the same family, the *Solanaceae*, the occurrence of precipitins in antiserum to virus-sap of tobacco common to the 4 plants is to be expected. The presence of antigenic substances in tobacco common to all of these different plants still leaves the possibility unaltered that the antigenic substance in virus-sap of tobacco, tomato, pepper, and petunia specific for antiserum to tobacco virus-sap, may be plant protein altered by disease. If the specific antigenic substance is foreign virus protein, for example, its antigenic property is apparently not altered by multiplication in different hosts.

In the hope of determining the presence of an antibody, specific for virus itself, a series of experiments were undertaken to test the power of inactivation of the antiserum to virus-sap on virus.

Inactivation Experiments with Virus-Sap and Antiserum.

Known amounts of antiserum were pipetted into tubes and varying amounts of freshly extracted virus-sap, diluted with saline solution (0.85 per cent), were added to the serum. The volume of liquid in each tube was made constant by the addition of saline solution. In 4 out of the 6 experiments carried out, the mixture of serum and virus-sap was held at room temperature for several hours; in the other 2 cases the mixture was kept for 1 hour at 37°C. (water-bath). Two different antisera to virus-sap, 2 to normal sap, and several samples of normal rabbit and guinea-pig serum were tested.

After the serum had remained in contact with the virus-sap for a given period, the contents of each tube were shaken thoroughly, poured into a dish and used for inoculation into young Turkish tobacco plants.

The inoculation of the plants was accomplished by scarifying 3 leaves on each plant with a sterile needle, dropping on the inoculum with a capillary pipette, and rubbing it in with a sterile cork. Every precaution was taken throughout the entire procedure to avoid accidental infection of the plants. Records of the occurrence of mosaic were made as the disease developed, for a period of 4 weeks, when the plants were discarded.

Control tests were included in which the virus-sap was placed in contact with antiserum to normal sap, normal rabbit and guinea-pig serum, and saline solution or tap water.

TABLE IV.

Summary of Results of Inactivation of Virus-sap with Antisera and Normal Serum.

Serum used for inactivation of virus	No. of individual mixtures of serum and virus-sap tested	No. of plants inoculated	No. of plants		Per cent of healthy plants
			Diseased	Healthy	
Active AV*	5	23	3†	20	86
(56°C.) AV	8	34	8‡	26	76
(56°C.) AV + alexin	7	32	3§	29	90
Total	20	89	14	75	88
Active AN	2	8	7	1	12
(56°C.) AN	1	6	3	3	50
(56°C.) AN + alexin	1	6	4	2	33
Total	4	20	14	6	30
Active normal	3	13	13	0	0
(56°C.) normal	1	6	6	0	0
(56°C.) normal + alexin	2	10	8	2	20
Total	6	29	27	2	6
Saline	6	26	26	0	0

* AV = antiserum to virus-sap.
AN = antiserum to normal sap.

† In 3 of 5 tests, complete inactivation occurred.

‡ In only 3 of 8 tests, complete inactivation occurred.

§ In 6 of 7 tests, complete inactivation took place. In the seventh instance, the corresponding amount of active serum also failed to produce complete inactivation.

In a series of experiments, the antisera to virus-sap and normal sap were drawn from the marginal ear-vein of the rabbit and allowed to stand on the clot overnight. The sera were tested on the following day in the active state, and after inactivation for one-half hour at 56°C., both with and without the addition of small amounts of fresh, normal, rabbit or guinea-pig serum (alexin).

The results of 6 separate experiments are summarized in Table IV.

Analysis of the Findings.—An examination of Table IV will show that:

Of 89 plants inoculated with virus-sap mixed *in vitro* with antiserum to virus-sap, 75 remained healthy.

The 26 corresponding control plants inoculated with the same amount of virus-sap in saline solution or tap water developed 100 per cent mosaic disease.

Of 20 plants inoculated with virus-sap mixed *in vitro* with antiserum to normal tobacco sap, only 6 remained healthy.

Twenty-seven of 29 plants inoculated with virus-sap mixed *in vitro* with normal rabbit or guinea-pig serum developed mosaic disease.

Discussion.—In respect to the power of inactivation of virus-sap exhibited by the antiserum to virus-sap, in comparison to that shown by antiserum to normal sap, and normal rabbit or guinea-pig serum, the results are so significant as to require no comment.

Several hundred plants were inoculated in these experiments, but since the concentration of virus varies with every preparation of virus-sap, the amount of antiserum required for complete inactivation cannot be determined in advance. The results of experiments in which a considerable excess of virus-sap was used are not reported in this paper. There is also some evidence to show that an excess of antiserum to virus-sap may inhibit an inactivation of the virus.

The results would seem to indicate further that antiserum to virus-sap, when heated for one-half hour at 56°C. (water-bath) loses some of its power to inactivate virus-sap (Table IV). The original strength of the antiserum can apparently be fully restored by the addition of rabbit or guinea-pig alexin. This behavior of the antiserum to virus-sap suggests that a lytic antibody for virus may be present in the antiserum. The importance of such a finding necessitates further efforts to corroborate these results.

Preliminary experiments indicate that the antiserum to virus-sap

of tobacco is also capable of inactivating virus-sap of tomato, pepper, and petunia plants.

SUMMARY.

1. Antisera were produced, separately, in rabbits to normal sap from healthy Turkish tobacco plants and to virus-sap from tobacco plants, affected with mosaic disease.

2. The immunologic reactions of the antisera were studied by means of:

(*a*) Alexin-fixation tests.

(*b*) Precipitation experiments, including: Precipitin-absorption tests with the same tobacco virus multiplied in tobacco, tomato, pepper, and petunia plants.

(*c*) Experiments with the inactivation properties of both antisera and normal serum on virus-sap.

3. From the results obtained from the above experiments, the following conclusions were drawn:

(*a*) Normal-tobacco-sap and virus-tobacco-sap possess antigenic substances in common.

(*b*) Normal sap and virus-sap of tomato, pepper, and petunia plants contain antigenic substances in common with normal sap of tobacco.

(*c*) Virus-saps of tomato, pepper, and petunia plants, have antigenic substances in common with virus-sap of tobacco, that are either not present in the normal tobacco sap or present only in small amounts.

(*d*) The two antisera possess alexin-fixing antibodies and precipitins in common.

(*e*) All of the precipitins to normal tobacco sap may be removed from either antiserum by absorption with virus-sap of tobacco.

(*f*) Specific precipitins for virus-sap of tobacco, tomato, pepper, and petunia are present in the antiserum to tobacco virus-sap and cannot be removed by complete absorption with normal sap of tobacco.

(*g*) Antiserum to virus-sap of tobacco, when used in an appropriate amount, has the power of completely inactivating virus-sap.

A corresponding quantity of antiserum to normal tobacco sap, or normal rabbit or guinea-pig serum, does not exhibit the same preventive action on virus-sap.

(*h*) There is some evidence that a specific antibody to virus-sap, lytic in nature, is present in the homologous antiserum.

In closing, the writer gratefully acknowledges the valuable criticism and suggestions offered by Doctor F. P. Gay and Doctor L. O. Kunkel in the course of these experiments.

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THE RELATION OF CHROMATIN TO HEMOGLOBIN AND BILIRUBIN.

By HERMAN H. RIECKER.

(From the Department of Internal Medicine, University Hospital, University of Michigan, Ann Arbor.)

PLATE 44.

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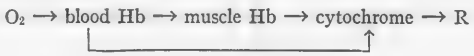
Recent studies of body pigment metabolism have given rise to two questions which await correlation. One is the origin of hemoglobin and the other that of the formation of bilirubin from other sources than hemoglobin. It is commonly accepted that blood pigment is the precursor of bilirubin, but Whipple (1) has suggested that hemoglobin and bilirubin may be formed independently of each other, probably in the liver, the former "by a progressive synthetic grouping of amino-acids, iron and other materials, which finally results in the finished product hemoglobin."

However there is evidence that hemoglobin appears in conjunction with the development of the erythrocyte and that it is derived from the chromatin of the hematopoietic cells. Schmidt (2), and more recently Sabin (3), have observed the presence of hemoglobin in the developing blood cells, although A. B. Macallum (4) first described its origin from the chromatin of the hematoblast in both amphibia and mammals.

Macallum devised methods of identifying cellular iron by microchemical methods and was able to distinguish the iron in hemoglobin from that in the chromatin of cells. His results showed that the first trace of hemoglobin in the earliest stages of an erythroblast was in the mitotic figure when the cell was dividing. These observations were based upon the fact that the iron in hemoglobin cannot be "unmasked" and specifically stained, while that of chromatin readily yields its iron in the presence of mineral acids.

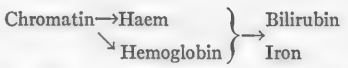
Since all the cells of the body contain chromatin, it is necessary to account for the fact that in these hemoglobin is not produced. Anson

and Mirsky (5) and Keilin (6, 7) have shown that all animal and vegetable cells (except anerobic bacteria) contain a respiratory pigment, called cytochrome, or haem. The function of this pigment is identical with that of hemoglobin and the following diagram illustrates Keilin's conception of the relation of cytochrome and hemoglobin to cell respiration in the body:



R being the oxidized substance in the cells.

The cytochrome or haem of the ordinary cell is then the analogue of the hemoglobin in the erythrocyte. It is logical to suppose that cytochrome is derived from the chromatin of the cells containing it and that on the death of the cell it yields bilirubin in the same manner as hemoglobin. A diagram illustrating this conception of the relation between the respiratory and bile pigments might be constructed as follows:



It is evident from this discussion that the theory of a primary "pigment complex" as expressed by Whipple (1) need not be postulated because chromatin itself wherever its location is probably the precursor of both hemoglobin and the respiratory pigment of the cells, and from these bilirubin is derived.

The relation of chromatin to hemoglobin formation could be strengthened further if it were possible to demonstrate a quantitative relationship between the rate of regeneration of hemoglobin to the chromatin content of the bone marrow cells in an animal with anemia due to hemorrhage. This possibility has been examined in work now to be described.

It was shown in a previous paper (8) that hemoglobin formation could be inhibited in an anemic animal by iron starvation. McMaster and Haessler (9) were able to show that body iron depletion causes a depression of red blood cell formation in the bone marrow. It follows then that by modifying the amount of iron available to the body cells one should be able to stimulate or inhibit bone marrow cell growth as well as hemoglobin formation.

Methods.

The general plan of study followed the methods previously described by Whipple and Robscheit-Robbins (10), dogs being used as experimental animals. The bone marrow was studied first, during a period of rapid blood regeneration and second, during a period of relative iron starvation, while the peripheral hemoglobin level was held stationary. Blood regeneration was stimulated by feeding iron citrate or raw liver in relatively large amounts.

In the study of the relation of iron to hemoglobin formation in the bone marrow the methods described by Macallum were followed in making microchemical stains of the bone marrow cells. The marrow from the femur was chosen as representative because the decalcification of rib made the specimen unfit for the staining of iron. In each instance marrow from several parts of the bone were prepared and sections were cut also from the spleen and liver. The fresh tissue is fixed in 95 per cent alcohol, sectioned, and then placed in a solution of mineral acid and 95 per cent alcohol. Nitric, hydrochloric or sulphuric acid may be used, preferably the latter in 4 per cent concentration. After a short period (12 to 24 hours depending on the nature of the tissue) the sections are stained by applying a mixture of freshly made ammonium sulphide, and glycerine to the tissue and heating this preparation at a temperature of 55° for several days. The iron containing parts of the cell will then appear green in color while any substance containing iron not capable of being unmasked in the process will remain unstained. Of the several methods of demonstrating iron in tissue, it appeared after considerable experimentation that the ammonium sulphide-glycerine method was the most suitable. The sections were also stained with an aqueous solution of 0.5 per cent hematoxylin but the clearing of the section permitted by this method did not compensate for the lack of uniformity which they presented owing presumably to an inconstant hydrogen ion concentration (11). The steps in the preparation of tissue for examination were as follows:

1. Fixation in 95 per cent alcohol 24 hours. 2. Absolute alcohol 12 hours. 3. Xylol and paraffin. 4. Sectioning (6 M thick). 5. Place on slide without adhesive. 6. After drying dissolve paraffin in xylol and place in acid alcohol 24 hours with sealed cover. 7. Wash in 95 per cent alcohol thoroughly. 8. Place one drop of ammonium sulphide and one drop of glycerine on preparation, mix with glass rod and seal with cover slip. 9. Place preparation in warming oven 4 days.

Seven dogs were used in the experimental work but only the protocols are given of those from which photographs were taken. They were fed a bread mixture consisting of starch, bran, tomatoes, wheat flour, sugar and yeast. The iron content of the bread mixture was found to be 0.0055 per cent. The dogs were allowed only distilled water, and during the last 7 to 10 days of a period in which it was desired further to restrict the iron intake only milk was given the animals. These steps insured a rather high degree of iron restriction.

All the dogs had been continuously used in other experiments for a year prior

to sacrificing, and the hemoglobin had been maintained at a low level during this period. Dog 4 was fed 300 gm. of raw beef liver daily until hemoglobin production had reached a maximum. The dog was killed and the bone marrow removed by splitting the shaft of the femur and removing the bone marrow intact for hardening. In Dog 10 the hemoglobin level was receding without the necessity for hemorrhage at the time the animal was sacrificed, while in Dog 13 ferric citrate was used to increase hemoglobin production before studying the iron content of the bone marrow.

TABLE I.

Rate of Regeneration of Hemoglobin When Bone Marrow Was Studied.

Date	Red blood cell count	Oxygen capacity	Hb	Blood drawn	Hb	Hematocrit, per cent cells	Wt.	Diet
			Dog 4					
1928	*millions*	*cc.*	*per cent*	*cc.*	*gm.*		*kg.*	
Feb. 20		10.4	56.3	20	1.5	29	12.9	Stock bread plus
" 23	6.54	11.1	60	20	1.5	30		raw beef liver
Mar. 1		10.6	57.3	100	7.9	30		300 gm. daily
" 5		11.7	63.3	100	8.7	32	13.0	
" 7								
			Dog killed					
			Dog 10					
Mar. 8		10.5	56.8	100	7.8	28	13.0	Whole milk only
" 15	5.52	9.5	51.4	15	—	24		
" 22		8.7	47.0	15	—	23		
" 22								
			Dog killed					
			Dog 13					
Jan. 4	6.09	9.2	50	20		26		Bread plus Fe
" 25		12.4	66	215	19.9	34		cit. 50 cc., 0.5
Feb. 6								per cent
			Dog killed					

The tissue blocks were cut at intervals during progress of the experiment, but staining procedures were carried out simultaneously on tissues on which comparisons of the staining reactions were to be made. The sections, usually four from each animal, were carried through the procedure to the warming oven at the same time, and when photographs were desired these were all made on the same morning for the entire set under identical conditions as to light, exposure, magnification, filters and development. Macallum found that the use of a steel blade in cutting

sections did not alter the staining depth although the handling of sections was carried out by means of glass rods always in glass containers. Study of individual cells was not undertaken since it was desired only to demonstrate the possible relation of rapid hemoglobin production to the amount of iron in bone marrow cells generally. It was necessary to observe in the sections that the green color due to iron was confined to the nuclei because any general diffusion of the color which occurred was evidence that the section was improperly prepared and must be discarded.

A direct estimation of the iron balance in dogs is impossible because of the large content of hair in the stools. However work in progress shows that a considerable depletion of the normal level of blood serum iron takes place under these conditions.

RESULTS.

The accompanying photomicrographs show the relative condition of the bone marrow with regard to its chromatin content. The sections demonstrated clearly that the iron content of the hematoblast is dependent on its chromatin content and that this in turn can be modified by iron feeding under the conditions of the experiments. In the animals in which the hemoglobin level is being barely maintained, nuclear chromatin stain is pale, while in those in which regeneration of hemoglobin was proceeding rapidly a heavy iron stain in the bone marrow is demonstrated.

The same phenomenon was present in sections from the spleen but in the liver the differences were not so manifest. As compared with the normal animal, however, all the tissues were poor in iron when anemia was present, and regardless of iron intake. Stains made of the liver and spleen of anemic dogs gave a much weaker chromatin reaction than those obtained from normal animals.

The interpretation of the depth of staining which different body cells exhibit after being subjected to the technique has been carefully considered. Since the chromatin is confined to the nuclei of the cells, the richness of its network is localized and the staining method itself prevents diffusion of iron into the surrounding tissues because water is not used in the preparation of the specimen. In order to further safeguard against mistakes in the subjective interpretation of the depth of staining only the two extremes in iron content were used. The bone marrow was either causing rapid production of hemoglobin or the hemoglobin level had remained stationary for a long period of

time under a constant stimulus. Hueck (12) has demonstrated a true comparison between the microchemical stain and chemical analysis of the tissues and his results have been confirmed in this laboratory by having a disinterested observer compare stained sections with the chemical analysis of tissues. The photomicrographs were made with the use of a red filter over a field large enough to include many cells.

The hypothesis that body cell chromatin, through cell metabolism, becomes the primary pigment complex is in no way antagonistic to the prevalent theories of pigment formation. It simply utilizes the facts already available and reduces the question to simpler and more basic considerations, whereupon investigation may continue.

SUMMARY.

1. Attention is directed to the diversity of opinion among investigators regarding the site and manner of hemoglobin formation in the body and its relation to bile pigment metabolism.

2. It is probable that in forming new hypotheses on this subject the earlier work of A. B. Macallum on the relation of chromatin to hemoglobin formation has not received sufficient consideration.

3. It has been shown by means of microchemical iron stains of the bone marrow cells, that the iron content of the hematoblast is increased during rapid hemoglobin production in simple anemia.

4. This fact is compatible with the work of Macallum who believed that hemoglobin is derived from the chromatin of the hematoblast. It does not support a theory that hemoglobin is formed as a part of a circulating pigment.

5. It is suggested that bilirubin is derived from the chromatin of body cells through the intermediary stages of the respiratory pigments, hemoglobin and cytochrome, from erythrocytes and other cells, respectively.

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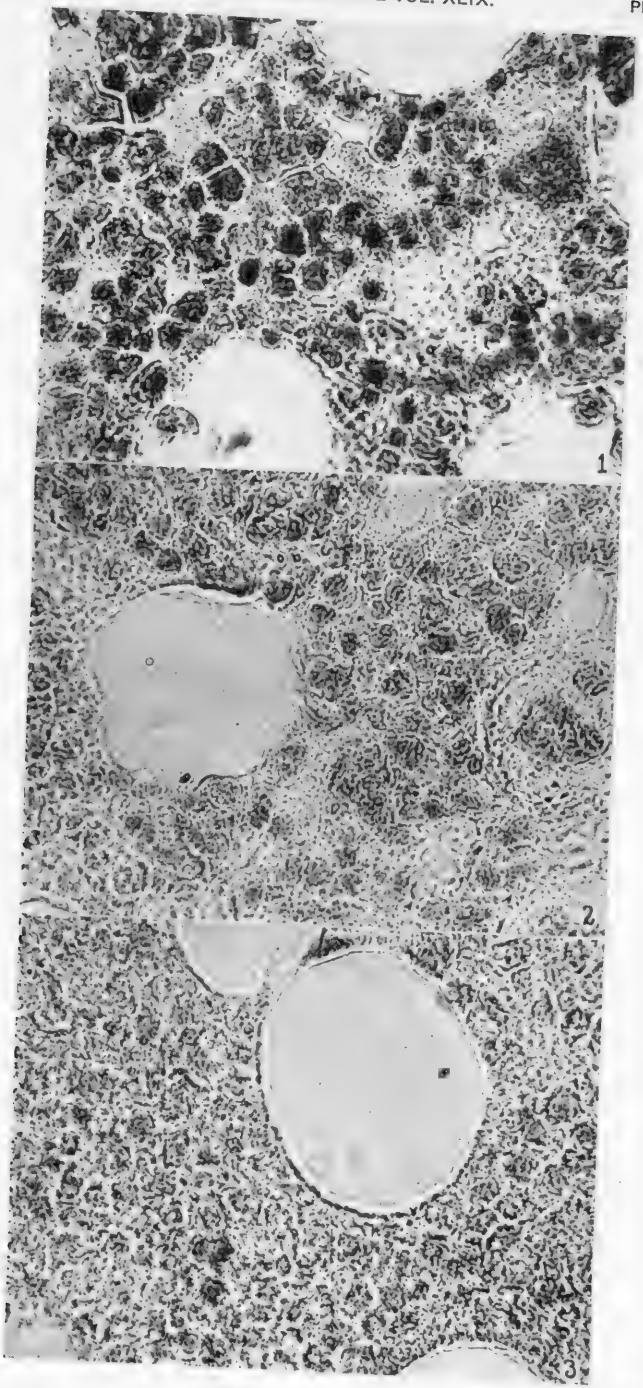
EXPLANATION OF PLATE 44.

FIG. 1. Dog 13, fed Fe citrate. Bone marrow, femur showing increased density of cells. Preparations cannot be cleared for photographs. Red filter used. Oil immersion with No. 10 ocular. Sulphuric acid alcohol 24 hours. Ammonium sulphide-glycerine.

FIG. 2. Dog 10, iron starved. Same as above showing lessened density of bone marrow cells.

FIG. 3. Dog 4, fed liver. Showing moderate increase in chromatin network.



(Riecker: Relation of chromatin to hemoglobin and bilirubin.)

EXPERIMENTAL DEHYDRATION: CHEMICAL CHANGES IN THE BLOOD OF THE DOG CONTRASTED WITH THOSE FOLLOWING OBSTRUCTION OF THE CARDIAC END OF THE STOMACH.

By RUSSELL L. HADEN, M.D., and THOMAS G. ORR, M.D.

(*From the University of Kansas School of Medicine, Kansas City, Kansas.*)

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Numerous factors are operative in the development of the toxemia characteristic of both simple and strangulation obstruction of the upper intestinal tract. Hartwell and Hoguet (1) were the first to emphasize the importance of dehydration and to show that life can be prolonged by supplying water. We now consider in every case of intestinal obstruction dehydration as well as other factors, such as hypochloremia. It is difficult to decide just what part anhydremia plays in the toxemia. One also does not know how far other factors may be dependent on the dehydration.

Experimental dehydration in the dog has been thoroughly studied over short periods of time by Keith (2). He found no marked changes in the urea nitrogen and carbon dioxide combining power of the plasma. The serum chlorides showed a constant increase averaging about 10 per cent. With the polyuria there was an increased urea and chloride output. On restoring the water balance sodium chloride was retained. Keith dehydrated his animals by injecting intravenously a 50 per cent solution of glucose or sucrose at the rate of 8 gm. per kilo of body weight over a period of 2 hours. Mackay and Mackay (3) made similar studies over a longer period on rabbits dehydrated with a hypertonic solution of sucrose. Blood chemical determinations on such animals from day to day showed a marked increase in blood urea. The rise in blood urea they ascribed largely to increased urea formation from accelerated tissue catabolism.

Dehydration as observed clinically in infants suffering from nutritional disturbances has been well studied by Utheim and others (4). The subject of anhydremia has been reviewed by Marriott (5). The characteristic features of dehydration are: an increase in viscosity, an increase in non-protein nitrogen due to both retention and accelerated protein destruction, decreased oxygen capacity, decreased blood volume and volume flow, impairment of renal function, a negative nitrogen balance, an increased destruction of body protein, and fever. Most of these

features are equally characteristic of upper gastro-intestinal tract obstruction. The increase in non-protein nitrogen was one of the first recognized chemical changes in the disease, and has been confirmed by all later workers (6). There is an increased nitrogen excretion (7) due to an increase in protein destruction. The viscosity is much increased (8) with necessarily an accompanying decrease in volume flow. The oxygen capacity is decreased (9) and renal function is impaired (10). Long ago Spiegler (11) and others demonstrated an increased nitrogen excretion after water deprivation due to accelerated protein breakdown.

The experiments herein recorded have been made to compare the changes observed in upper gastro-intestinal tract obstruction with those of simple dehydration and determine in how far the chemical changes in obstruction might be explained by dehydration alone. The dehydration was effected by making daily one or more injections of a 50 per cent sucrose solution, 8 cc. per kilo of body weight being given at each injection. Urine collections are most difficult to make in intestinal or pyloric obstruction. We have shown (12) that dogs with obstruction of the cardiac end of the stomach develop a typical toxemia even more marked than with intestinal obstruction. Such animals cannot vomit so urine collections can be satisfactorily made. For this reason the results in a series of such animals are compared with those of a group dehydrated with sucrose.

Method.

All operations were done under ether anesthesia with aseptic technique. The obstruction of the cardia was effected by ligating with tape. All injections were made slowly into the jugular vein. The non-protein nitrogen was determined by the method of Folin and Wu (13), the urea nitrogen by the Van Slyke and Cullen modification of the Marshall method (14), the CO_2 combining power by the method of Van Slyke and Cullen (15), and the chloride on the tungstic acid filtrate in the manner suggested by Gettler (16). The hematocrit reading was obtained by centrifuging 10 cc. of blood mixed with 2 cc. of 1.6 per cent sodium oxalate (17) at high speed for ½ hour. The fibrin determinations were made on this oxalated plasma by the method of Foster and Whipple (18). The total protein of the serum was determined by the micro Kjeldahl method. The globulin was precipitated with 22.2 per cent sodium sulphate as suggested by Howe (19) and the albumen determined in the filtrate. The globulin was obtained by difference.

EXPERIMENTAL OBSERVATIONS.

The chemical findings in the blood and urine of five animals dehydrated with sucrose are shown in Table I. The animals survived three

to five days, the average duration of the experiment being 3.8 days. Four animals showed a terminal rise in non-protein and urea nitrogen. The CO_2 combining power showed little change. The blood chlorides were uniformly elevated, the average increase being 13.9 per cent. The fibrinogen content of the plasma was increased in all animals. The total protein of the serum showed an average increase of 18 per cent. This increase was largely in the albumen fraction. The plasma percentage as determined in the hematocrit tube showed a decrease in all animals although in three animals there was a terminal increase. The average output of urine was 1102 cc. for the duration of the experiment. The nitrogen output was variable. The chloride excretion was usually high.

The findings in five animals with obstruction of the cardiac end of the stomach are shown in Table II. All animals showed the characteristic rise in non-protein nitrogen and urea nitrogen. The CO_2 combining power showed little change. In three animals there was a slight fall in chlorides and in two a slight rise. The fibrinogen content of the plasma is uniformly markedly increased. The total protein of the serum shows in every animal a marked increase. This increase affects both the globulin and albumen fractions. The plasma percentage is decreased. All animals showed a diuresis with a rather high nitrogen and salt output.

DISCUSSION.

The comparative data in dehydration and in obstruction of the cardiac end of the stomach are summarized in Table III. Several significant facts are evident. The increase in non-protein nitrogen and urea nitrogen is much the same in the two groups, although slightly higher in obstruction with a shorter average duration of life. The chlorides show the characteristic rise with dehydration. There is a slight fall with obstruction. The fibrinogen figures also show a marked difference in the two groups. With dehydration the average final determination is approximately double that of the initial determination; with obstruction the fibrinogen value is approximately quadrupled in a shorter time. The average protein increase with dehydration is 18.6 per cent, with obstruction it is 33 per cent. The final

TABLE I.

Chemical Findings in Blood and Urine of the Dog After Dehydration with Sucrose (8 cc. per kilo body weight of 50 per cent solution at each injection).

Dog No.	Day of experiment	Weight	Blood (amount per 100 cc.)									Urine					Remarks
			Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Fibrinogen	Globulin (serum)	Albumin (serum)	Total protein (serum)	Plasma volume	Amount	Chlorides		Nitrogen		
		kilo	*mg.*	*mg.*	*mg.*	*vol. per cent*	*mg.*	*gm.*	*gm.*	*gm.*	*cc.*	*cc.*	*per cent*	*gm.*	*per cent*	*gm.*	
6-54	1	9.0	30.6	14.9	460	32.4	875	2.41	3.66	6.06	56	550	0.24	1.32	0.72	3.96	2 injections
	2		27.7	15.9	490	30.5	930	2.41	3.87	6.30	47	250	0.14	0.35	0.23	0.58	2 "
	3	8.3	80.8	43.9	530	31.5	1250	2.51	4.36	6.86	47	100	0.25	0.25	0.21	0.21	1 "
	4																Dead
6-55	1	10.6	33.0	5.1	450	27.7	700	2.62	3.92	6.54	53	600	0.36	2.16	0.29	1.74	1 injection
	2	10.2	30.9	10.3	470	28.7	822	2.82	3.81	6.63	56	600	0.40	2.40	1.50	9.00	2 "
	3	9.6	30.0	10.3	490	28.7	1494	3.00	4.12	7.19	53	120	0.18	0.22	0.55	0.66	1 "
	4		33.0	18.6	480	30.5	1803	3.08	4.36	7.44	49	244	0.15	0.37	0.60	1.40	1 "
	5	8.9	68.3	37.4	320	33.4	2332	3.53	4.51	8.05	59	104	0.06	0.06	2.9	3.02	2 "
	6																Dead
6-56	1	8.2	27.6	7.5	490	32.4	1153	2.01	3.71	5.70	59	400	0.31	1.24	1.23	4.92	1 injection
	2	7.8	27.2	10.7	490	28.7	1141	2.12	3.85	5.97	55	400	0.30	1.20	0.38	1.52	2 "
	3	7.1	50.0	28.0	480	40.0	1659	2.65	4.94	7.59	51	84	0.10	0.08	0.35	0.29	1 "
	4	6.7	118.0	77.1	570	25.6	2612	2.21	4.33	6.55	59	90	0.23	0.21	0.80	0.72	1 "
	5																Dead

6-59	1	13.6	43.6	20.6	500	41.9	938	3.86	3.85	6.71	56	650	0.81	5.30	0.96	6.24	1 injection
	2		43.9	22.4	520	43.8	1112	2.66	4.09	6.75	56	308	0.30	0.92	0.70	2.16	1 "
	3	11.1	93.7	40.6	560	32.4	1571	3.43	4.38	7.81	48	150	0.58	0.87	1.92	2.88	1 "
	4	10.8	103.0	76.6	550	25.8	1822	3.45	4.88	8.34	56	0					Died soon after
6-60	1	11.2	33.7	10.27	490	29.6	943	2.18	3.40	5.63	55	774	0.74	5.70	0.96	7.73	1 injection
	2		27.8	14.9	510	32.4	872	2.03	3.61	5.65	54	86	0.34	0.29	2.80	2.41	1 "
	3	10.5	33.3	16.8	550	29.6	1028	2.35	4.21	6.56	54	0					Died soon after

TABLE II.

Chemical Findings in Blood and Urine of the Dog After Obstruction of the Cardiac End of the Stomach.

Dog No.	Day of experiment	Weight	Blood (amount per 100 cc.)									Urine					Remarks
			Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Fibrinogen	Globulin (serum)	Albumin (serum)	Total protein (serum)	Plasma volume	Amount	Chlorides		Nitrogen		
		kilo	*mg.*	*mg.*	*mg.*	*vol. per cent*	*mg.*	*gm.*	*gm.*	*gm.*	*cc.*	*cc.*	*per cent*	*gm.*	*per cent*	*gm.*	
6-61	1	16.0	30.3	12.1	520	32.4	651	3.9	4.0	7.20	54	110	0.90	0.99	0.41	0.45	Operation
	2		71.0	43.9	480	31.5	1398	4.11	5.64	9.79	45	0					Died soon after
6-62	1	10.0	31.6	19.6	440	28.7	600	2.71	4.80	7.51	42	870	0.51	4.44	0.41	3.57	Operation
	2		67.5	40.6	440	32.4	1112	2.33	5.71	8.03	35	92	1.01	0.93	1.32	1.21	
	3		163.0	82.2	450	27.7	2031	2.38	6.32	8.66	35	0					
6-63	1	10.1	28.9	14.0	430	40.0	571	1.78	4.73	6.51	48	260	0.57	1.48	3.78	9.83	Operation
	2		37.5	13.1	500	44.7	958	2.15	5.01	7.18	37	190	0.62	1.18	1.65	3.14	
	3		42.0	12.1	440	34.3	1993	1.87	5.46	7.35	34	260	0.34	0.88	0.25	0.65	
	4		114.0	71.9	410		2739	2.63	5.75	8.39	33	0					
6-65	1	9.5	31.9	11.2	450	34.3	923	3.55	3.56	6.91	58	480	0.89	4.27	0.62	2.98	
	2		37.7	19.6	450	40.0	1606	4.23	4.20	8.43	46	200	0.69	1.38	0.38	0.76	
	3		70.5	39.7	470	34.3	2270	4.45	5.02	9.46	45	300	0.39	1.17	0.78	2.28	
6-69	1	9.0	26.1	12.6	470	36.2	1158			6.28	50	130	0.66	0.86	2.32	2.99	Operation
	2		26.9	7.5	460	36.2	2068			7.47	42	170	0.51	0.87	1.65	2.89	
	3		63.4	27.8	460	39.0	3465			9.43	50	0					

TABLE III.

Comparative Findings in the Blood and Urine of the Dog After Dehydration with Sucrose and in Obstruction of the Cardiac End of Stomach.

Experiment	Average weight	Number of experiment	Average duration of experiment	Blood (amount per 100 cc.)														Urine (average)	
				Non-protein nitrogen		Urea nitrogen		Chlorides (as NaCl)		CO_2 combining power		Fibrinogen (plasma)		Total protein (serum)		Plasma volume		Total amount	Per kilo
				Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final		
	kilo		*days*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*vol. per cent*	*mg.*	*mg.*	*gm.*	*gm.*	*cc.*	*cc.*	*cc.*	*cc.*
Dehydration with sucrose...	10.5	5	3.8	36.1	80.7	11.7	50.4	478	544	32.8	29.2	922	1809	6.13	7.27	56	57	1102	105
Obstruction of cardiac end of stomach.....	10.9	5	3.0	29.8	96.4	13.9	53.1	462	454	34.2	32.8	581	2381	6.88	9.15	50	42	621	57

plasma volume is less with obstruction than with dehydration. The average total urine output for the duration of the experiment is 1102 cc. or 105 cc. per kilo with dehydration as contrasted with 621 cc. or 57 cc. per kilo in obstruction. At autopsy the stomach and intestinal tract of the animals with obstruction of the cardia contained only a minimal amount of fluid.

These results show that with a much less marked anhydremia the animals with obstruction of the cardiac end of the stomach present a much more marked chemical evidence of toxemia than animals simply dehydrated with sucrose. These facts indicate that there must be some factor besides dehydration in the characteristic toxemia of cardiac obstruction. This generalization must apply also to pyloric and high intestinal obstruction.

SUMMARY AND CONCLUSIONS.

A comparative chemical study of the blood and the urine of the dog with experimental dehydration and with obstruction of the cardiac end of the stomach is reported.

The average duration of life is slightly longer with dehydration than with obstruction.

The urine output per kilo of body weight is almost twice as great in dehydration as with obstruction.

The increase in non-protein nitrogen and urea nitrogen is much the same in the two groups although somewhat more marked with obstruction.

The chlorides of the blood are markedly increased with dehydration and slightly decreased with obstruction.

The increase in fibrinogen and total protein is twice as great with obstruction as with dehydration.

These findings indicate that there must be some factor or factors in addition to dehydration producing the toxemia of cardiac obstruction.

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CHEMICAL FINDINGS IN THE BLOOD OF THE DOG AFTER CLOSED-LOOP OBSTRUCTION OF THE JEJUNUM.

By RUSSELL L. HADEN, M.D., and THOMAS G. ORR, M.D.

(*From the University of Kansas School of Medicine, Kansas City, Kansas.*)

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The chemical changes in the blood of man and the experimental animal after simple intestinal obstruction are well established. There is typically a rapid fall in chlorides, usually a coincident rise in the CO_2 combining power, and a later increase in the non-protein nitrogen and urea nitrogen (1). In such an obstruction there is probably no absorption of toxic bodies, the product either of bacterial action or of digestive activity, from the lumen of the intestine. However, there may be a disordered function of the mucosa.

In strangulation obstruction other possible factors in the characteristic toxemia are added to those encountered in simple obstruction. As a result of the interference with the blood supply of the obstructed loop toxic bodies from the autolysis of the strangulated tissue as well as from bacterial and digestive activity may be absorbed. Wangensteen and Waldron (2) have shown that the products of the autolysis of the small intestine are very toxic. Mann (3), and Mason and Davidson (4) had previously demonstrated a marked difference in the toxicity of the products of autolysis of different organs when allowed to autolyze within the abdominal cavity. Liver is very toxic while the spleen is relatively little so. Whipple (5) has shown also the very marked toxic effect of the autolyzing mucosa of the small intestine after exposure to the x-ray.

The experiments recorded here have been made to compare the chemical findings in the blood of the dog with closed-loop obstruction of the upper jejunum with those characteristic of simple obstruction at the same level. In these experiments there is no interference with the blood supply. With such closed loops there is the best of opportunity for the absorption of the products of digestive and bacterial activity due to the pressure which always develops as the loop dis-

TABLE I.

Chemical Findings in Blood of Dog with Closed-Loop Obstruction of Jejunum.

Dog No.	Day after operation	Blood			CO_2 combining power
		Amount per 100 cc.			
		Non-protein nitrogen	Urea nitrogen	Chlorides (as NaCl)	
		mg.	*mg.*	*mg.*	*vol. per cent*
4-93	0	28.2	14.0	490	39.0
	1	118.0	58.8	380	50.2
5-04	0	20.2	9.8	480	36.2
	1	20.3	10.5	410	41.9
	2	20.3	10.5	450	34.3
	3	34.9	17.7	350	38.1
	4	74.3	37.8	270	—
5-18	0	32.6	17.5	520	36.2
	1	40.0	25.2	460	43.8
	2	41.9	23.1	450	42.8
	3	238.0	127.2	440	40.0
	4	234.0	125.3	320	—
5-17	0	36.6	19.6	410	53.9
	1	37.5	19.6	320	44.7
	2	77.3	36.4	330	25.6
	3	152.0	82.0	320	28.7
4-96	0	22.4	11.2	470	38.1
	1 a.m.	36.9	19.6	390	48.3
	p.m.	36.9	18.6	350	45.7
	2 a.m.	45.7	25.9	320	52.0
	p.m.	78.0	40.6	300	60.5
	3 a.m.	84.3	42.0	290	65.3
	p.m.	205.0	102.2	270	65.3
	4 a.m.	246.0	121.2	220	67.1
	p.m.	246.0	122.6	220	68.1
4-95	0	20.2	4.2	460	38.1
	1 a.m.	43.6	21.7	400	43.8
	p.m.	48.9	24.5	410	44.7
	2 a.m.	45.3	23.1	370	50.2
	p.m.	58.0	29.4	330	50.2
	3 a.m.	103.0	75.7	250	38.1

tends. Almost all animals also show quite marked necrosis of the mucosa. This type of obstruction has, of course, no clinical analog.

Method.

All operations were done under ether anesthesia with aseptic technique. A loop of upper jejunum 8 to 10 inches in length was isolated and the ends closed by inverting and suturing the closed ends. The continuity of the intestine was restoring by making a lateral anastomosis. The animals were allowed water *ad libitum* but were given no food during the course of the experiment. Blood was obtained from the jugular vein for chemical analysis before operation and at 12 or 24-hour intervals until the end of the experiment. The non-protein nitrogen was determined by the method of Folin and Wu (6), the urea nitrogen by the Van Slyke and Cullen modification of the Marshall method (7), the CO_2 combining power by the Van Slyke and Cullen method (8), and the chlorides on the tungstic acid filtrate in the manner suggested by Gettler (9).

EXPERIMENTAL OBSERVATIONS.

The blood chemical findings in 6 dogs with closed-loop obstruction are shown in Table I. All animals showed the changes typical of simple high intestinal obstruction. The fall in chlorides is well marked in all. The increase in non-protein nitrogen and urea nitrogen which takes place in intestinal obstruction in a very short time is well shown in this series of experiments. The non-protein nitrogen of the blood of Dog 4-93 rose from 28.2 mg. per 100 cc. to 118 mg. within 24 hours after the obstruction was made. In Dog 4-96 the non-protein nitrogen increased from 84.3 mg. to 205 mg. per 100 cc. between the morning and afternoon determinations. Such increases can be explained only on the basis of a great acceleration of protein destruction.

At autopsy the closed loop was always markedly dilated, and usually discolored. In some there was marked destruction and desquamation of the mucosa of the loop. No animals are included in which there was peritonitis or evidence of leakage from the loop. Usually there was a great deal of vomiting from the onset of the experiment. In each animal the lateral anastomosis was functioning so the continuity of the lumen was assured.

DISCUSSION.

These results only emphasize that the changes characteristic of simple intestinal obstruction also take place when a closed intestinal

loop is made with a lateral anastomosis for resumption of continuity. The increase in non-protein nitrogen is rapid and most marked. The animals live a shorter time than with simple obstruction. The increased absorption and possibly disordered function of the mucosa which must take place, only accelerate the toxemia and the characteristic chemical changes.

SUMMARY AND CONCLUSIONS.

The chemical findings in the blood of 6 dogs with closed-loop obstruction of the upper jejunum are reported.

The duration of life with closed loops is less than with simple obstruction.

All animals showed a marked rise in non-protein nitrogen and urea nitrogen, and fall in chlorides. Usually the CO_2 combining power of the plasma is increased.

The findings in closed-loop obstruction are essentially the same as in simple intestinal obstruction.

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INTRADERMAL VERSUS SUBCUTANEOUS IMMUNIZATION OF MONKEYS AGAINST POLIOMYELITIS.

By F. W. STEWART, M.D., and C. P. RHOADS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Early experiments of Flexner and Lewis (1), Levaditi and Landsteiner (2), and Römer and Joseph (3) showed that monkeys once recovered from poliomyelitis are immune to subsequent intracerebral inoculations of poliomyelitis virus. This immunity was apparent no matter how slight had been the symptoms of the initial infection. The refractory state was of long duration and was absolute within the limit of infecting doses employed. These same workers noted that sera of convalescent monkeys, when mixed with poliomyelitis virus *in vitro*, rendered the material, otherwise infective, inactive when introduced intracerebrally in test animals.

These observations became the basis for numerous efforts to immunize animals against experimental poliomyelitis. Flexner and Lewis (4) injected monkeys subcutaneously with living active virus, beginning with a dose of 0.05 cc. This amount was given daily for four days and the series was repeated twice with a four day rest period between each individual set of injections. After the last interval the animals received on successive days 0.1, 0.5, and 1.0 cc. of virus, and after one month 5.0 cc. A week's rest period then followed, after which time the animals were tested intracerebrally with 2.0 cc. of fresh Berkefeld filtrate of poliomyelitis virus. Control monkeys which received 0.1 to 0.01 cc. of a similar filtrate intracerebrally developed typical poliomyelitis, whereas the vaccinated animals remained free from symptoms.

Levaditi and Landsteiner (5) attempted to immunize monkeys by a single subcutaneous inoculation of 0.5 cc. of virus suspension previously heated to 50°C. for 30 minutes. They failed to produce any immunity by this treatment. In another experiment glycerinated virus was heated to 50°C. for 2 hours; this heated virus was still active in producing disease when inoculated intracerebrally, but did not infect when given daily in subcutaneous doses of 2 cc. each over a period of one month. Nine days after the last subcutaneous injection two treated monkeys were tested intracerebrally. One of these showed slight prodromal symptoms of

poliomyelitis and the second gave no evidence of disease, whereas the control developed typical poliomyelitis.

Kraus (6) attempted the attenuation of poliomyelitis virus by phenolization and found that virus treated by 1 per cent phenol was rendered ineffective in four days, even when inoculated subdurally. He then endeavored to immunize monkeys by subcutaneous injection of 5 to 10 cc. of virus treated with varying concentrations of phenol for different periods of time. Of fifteen animals subjected to intracerebral test inoculations, twelve were immune. It is interesting to note that three animals immunized with virus treated with 1.5 per cent phenol for five days—a procedure calculated by Kraus to render virus inactive—were completely protected. In a second communication Kraus (7) reports results on two animals, one of which received 5 cc. of fresh virus cord emulsion subcutaneously, followed fifteen days later by 6 cc. of 0.5 per cent phenolized cord; a second monkey received 6 cc. of 0.5 per cent phenolized cord. Ten days later both monkeys resisted a test intracerebral inoculation with paper filtrate, whereas a control developed the typical disease.

Olaf Thomsen (8) gave monkeys daily sub-infective inoculations subcutaneously for twelve days and subsequently at weekly intervals, 0.06, 0.2, 0.4, 1.0, and 2.0 cc. of virus suspension. All animals were then resistant to intracerebral test but the author states that every animal showed symptoms such as excitement, tremor, and ataxia, during immunization. A second group was treated, using considerably smaller immunizing doses; of this series no animal showed symptoms during the immunizing procedure, yet all resisted test inoculation. The initial immunizing dose in this second series was only one hundredth of the estimated intracerebral infecting dose.

Zappert, Wiesner, and Leiner (9) attempted to immunize four monkeys by means of subcutaneous injections of gradually increasing doses of active virus emulsions. During the immunization, two of the animals died of intercurrent infection, one of typical poliomyelitis, and one of a supposedly marantic type of the disease. They attempted to induce an artificial immunity in one animal by the use of phenolized virus. The monkey developed the disease during the treatment designed to immunize against it.

Flexner and Amoss (10) described a so-called immunizing strain of poliomyelitis virus. To free a contaminated glycerinated brain from organisms, the tissue was immersed in 0.5 per cent phenol for a few hours and replaced in glycerine. This procedure was repeated once. The animals were subsequently infected with 1.0 cc. of 10 per cent suspension, their disease running an average eleven day course. In a series of passages the virulence of the strain decreased until a point was reached where the monkeys showed very few symptoms,—such as ataxia, tremor, and slight convulsive seizures—recovered, and were subsequently immune to strong virus.

Abramson and Gerber (11) treated emulsions of brain and cord of poliomyelitic monkeys for four hours with 0.5 per cent formaldehyde; this material was infective

when introduced subcutaneously in monkeys. They then endeavored to immunize by heated virus. On five successive days monkeys were injected with cord emulsion; the emulsion was heated to 55°C. for thirty minutes the first and second day, to 45°C. for thirty minutes the third day, to 37°C. for thirty minutes the fourth, and was used without preliminary heating on the fifth day. The dose was 5 cc. on each day. Three weeks after treatment the animals were bled and their sera tested for its power to neutralize virus. Of eight sera, three neutralized, four led to prolonged incubation period, and one failed. Intracerebral tests indicated that five of the treated monkeys were resistant to three to six minimum lethal doses of virus, whereas three proved susceptible.

In another series of three monkeys, Abramson and Gerber gave daily injections subcutaneously of 5 cc. of 10 per cent cord emulsion previously heated to 55°C. for one hour. On intracerebral test three weeks later, all developed poliomyelitis; of the three sera tested, one monkey showed no symptoms and two a delayed incubation period but eventually developed the disease.

McKinley and Larson (12) inoculated monkeys intracerebrally with 0.15 cc. of filtrate of a mixture of 5 per cent emulsion of castor oil soap and virus emulsion. The animals remained well and later resisted intracerebral inoculation of 0.7 cc. virus filtrate. Four more monkeys received 4 cc. of the virus-soap mixture intraperitoneally; none developed poliomyelitis, whereas a control with virus alone became paralyzed in a typical manner. Eleven days after the intraperitoneal virus-soap treatment, all four monkeys were tested intracerebrally; three remained well and one developed poliomyelitis.

The largest and most varied series of tests of poliomyelitis immunization is that of Aycock and Kagan (13). These investigators attempted to immunize with virus attenuated by various methods. The old experiments of Kraus with phenolized virus were repeated using material treated with 1.0, 0.75, 0.50, and 0.25 per cent phenol. The mixtures were kept for seven days in the icebox. Monkeys were then given four injections every other day of from 8 to 10 cc., beginning with the 1.0 per cent phenolized virus, and ending with the 0.25 per cent. Of four animals so treated, two became paralyzed during the process of vaccination, one failed to resist intracerebral test inoculation, and one resisted. In a second experiment monkeys were injected subcutaneously with virus cords dried over caustic potash from one to twenty-six days. Two of six monkeys became paralyzed during treatment, two failed to show protection on intracerebral inoculation, and two proved resistant. Next, virus cord was exposed to different glycerol-water dilutions (5 to 50 per cent glycerol) for seven months at ice box temperature. Monkeys were injected daily subcutaneously, beginning with virus from 5 per cent glycerol and ending with 50 per cent glycerol. Three animals developed paralysis during immunization; three failed to resist an intracerebral test; one resisted. In another group, virus in agar was introduced subcutaneously in eight animals; the total virus emulsion given ranged from 20 to 96 cc. of 5 per cent suspension in

from three to seventeen injections; two animals became paralyzed during treatment, two failed to show subsequent immunity, and two resisted.

In a fifth experiment virus was introduced intracutaneously in from 1 to 2 cc. amounts but was distributed in 0.05 cc. blebs, thus making from twenty to forty piqures each day of inoculation. The total amount of virus injected ranged from 5 to 76 cc. in six to forty-three inoculations, given during a period ranging from fifteen days to five months. Twelve monkeys were used; one became paralyzed during treatment; one failed to resist intracerebral inoculation; ten resisted one intracerebral test, but of these, two failed to withstand a second such test. Serum from eight resistant monkeys neutralized virus twenty-one times; one monkey's serum protected in one test, although the animal itself was not immune to intracerebral test inoculation.

From the review of the literature, it is apparent that the results of experiments designed to immunize monkeys against poliomyelitis have been inconclusive. Two facts stand out clearly; first, that it is impossible to protect monkeys by the use of killed virus, and second, that a definite though inconstant resistance to poliomyelitis can be brought about by the intradermal and subcutaneous introduction of the living virus. It was therefore deemed advisable to compare the results of the two routes of inoculation in order to gain information as to their relative efficacy. The following experiments were carried out with this point in view.

Experimental.

Eight monkeys (Table I) were immunized by the intracutaneous route, following in general the procedure of Aycock and Kagan. The injections were made biweekly and the total amount of a single day's dosage (1.5 to 2.0 cc. of 5 per cent glycerolated virus) was distributed in some twenty small blebs. The duration of the immunizing period was variable, lasting from three to five months. The total amounts of virus administered ranged from 42 to 66 cc. Before intracerebral test inoculation, all animals were bled in order to test their sera for virus-neutralizing power. The test inoculations were made with fresh virus injected intracerebrally in doses of 0.5 cc. of 5 per cent suspension. During the immunization period all animals were observed daily in order to detect possible abortive symptoms of disease and were exercised to bring out masked weaknesses.

Eight more monkeys (Table II) were treated in an analogous fashion but received their immunizing virus subcutaneously instead of intracutaneously. The amounts of virus used and the time intervals were comparable with those of the intracutaneous series, and bleedings and test inoculations were done in the same manner. Both tests for active immunity and for passive serum protection were rigorously controlled. The results in the two series are best seen in the tables.

DISCUSSION.

The primary purpose of this series of experiments was to determine whether the intradermal or the subcutaneous introduction of poliomyelitis virus was most effective in protecting monkeys against virus inoculation. Reference to Tables I and II shows that the degree of immunity produced is strikingly in favor of the intradermal method. Of the eight animals subjected to that procedure, all but one showed slight symptoms of the disease when tested by intracerebral inoculation of an amount of virus sufficient to cause characteristic poliomyelitis in the controls. No animal, however, developed more than the mildest abortive symptoms, such as tremor or excitement. No definite paralysis developed in any instance, and no subsequent muscle atrophy was observed. These results are sharply at variance with those of intracerebral inoculation of the group of monkeys treated by subcutaneous inoculation of virus. Four of the eight animals of this series developed typical poliomyelitis which progressed to prostration in two instances, and to well-marked paralysis in the other two. The remaining four animals proved to be completely refractory to the intracerebral tests.

During the process of immunization, the animals were closely observed to determine whether or not they developed an abortive form of poliomyelitis which might explain the subsequent immunity to the disease. Wickman (14), during the Swedish epidemic of 1905, noticed a considerable number of cases in man, which showed slight, transient symptoms, without developing the outspoken disease. Caverly (15), in the Vermont epidemic of 1904, saw six children with fever, nausea, and convulsions, whose illness never progressed further. Medin (16) also observed such abortive cases. Aycock (17) mentions the possibility that mild attacks of poliomyelitis are responsible for the development of immunity. In view of these observations we were on the alert to detect slight symptoms referable to the treatment. However, no deviation from the normal was discovered. Subcutaneous inoculation of virus has in our experiments on eight animals failed to produce the disease, although it has given rise to poliomyelitis in the hands of others (Flexner and Lewis, Aycock, and Olaf Thomsen). As evidence of the relative safety of intradermal inoculation of virus, in experiments to be reported, as much as 16 cc. of virus suspension

TABLE I.

Intradermal Immunization.

Monkey	Immunization		Total virus inoculated, 5 per cent suspension	Strain	Intracerebral test			Result of test		Serum neutralization	
	Begun	Ended			Date	Amount	Strain	Tested animal	Control	Test	Control
			cc.								
1	9/21/27	12/27/27	42	M.A.	Died of tuberculosis during immunization						
2	9/21/27	1/19/28	66	M.A.	1/19/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms	Typical poliomyelitis Prostrate in 6 days	Pooled neutralized	Typical poliomyelitis Prostrate in 11 days
3	9/21/27	1/ 3/28	48	M.A.	1/19/28	0.5 cc. 5 per cent suspension	M.A.	Slight excitement			
4	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Slightly slow	Typical poliomyelitis Prostrate in 6 days	Neutralized	Typical polio
5	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Tremor and ataxia; weak deltoid		Neutralized	Typical polio
6	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Slightly slow		Neutralized	Typical polio

7	6/ 8/28	11/ 1/28	56	Aycock	11/20/28	0.5 cc. 5 per cent suspension	M.A.	Slow and excited	Typical poliomyelitis Paralyzed in 8 days	Neutralized	Typical polio
8	6/ 8/28	11/ 1/28	56	Aycock	11/20/28	0.5 cc. 5 per cent suspension	M.A.	Excitement and tremor		Neutralized	Typical polio

TABLE II.

Subcutaneous Immunization.

Monkey	Immunization		Total virus inoculated, 5 per cent suspension	Strain	Intercerebral test			Result of test		Serum neutralization	
	Begun	Ended			Date	Amount	Strain	Test animal	Control	Test	Control
			cc.								
9	10/27/27	1/27/28	45.9	M.A.	2/11/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms	Prostrate 9th day	Pooled neutralized	Paralyzed 17th day
10	10/27/27	1/27/28	42.8	M.A.	2/11/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms			
11	10/27/27	1/27/28	38	M.A.	2/11/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms			
12	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Prostrate 10th day	Prostrate 11 days	Neutralized	Typical polio
13	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Paralyzed 18th day. Recovered		Serum contaminated	
14	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Prostrate 9th day		Typical polio	

15	6/ 8/28	10/16/28	56	Aycock	11/20/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms	Prostrate in 7 days	Not done	
16	6/ 8/28	10/16/28	56	Aycock	11/20/28	0.5 cc. 5 per cent suspension	M.A.	Paralyzed on 9th day. Recovered		Neutralized	Typical polio

has been given intracutaneously at one time without producing symptoms, while 0.005 cc. of Berkefeld filtrate of virus of the same strain inoculated intracerebrally consistently produced characteristic poliomyelitis in six days.

The question of the degree of protection conferred by the treatment proved to be an extremely interesting one. It has often been observed that different strains of poliomyelitis virus vary markedly in their power to produce the disease in susceptible animals. We therefore attempted to detect degrees of immunity by testing animals by intracerebral inoculation, not only with virus of the strain with which they had been immunized, but also with other strains. Thus monkeys treated with the M.A. strain of virus were tested with a fairly recent virus isolated in Vermont by Aycock, and animals immunized with Aycock strain were tested with the M.A. virus. The difference in the results is well-marked; monkeys treated in exactly the same way proved totally resistant to the relatively weak M.A. virus and not totally immune to the stronger Aycock virus. A group of three animals immunized with M.A. strain is described in Table III. All withstood subsequent intracerebral inoculation with both M.A. and Aycock virus but one of the three developed typical poliomyelitis on inoculation with a very active pooled, mixed virus derived from material of the original M.A. and K. strains which had been preserved in glycerol since 1920 (18, 19, 20).

That the immunity induced in the monkeys in these experiments is relative only, is more strikingly shown by tests employed to determine the power of the sera to neutralize the virus. The results of these determinations are shown in Table IV. The sera of Monkeys 1, 2, and 3 were pooled in one and those of 9, 10, and 11 in a second group. These two mixed sera neutralized, as was to be expected, since on intracerebral test the monkeys had proved resistant. Moreover, Sera 6, 7, and 8, derived from monkeys which had presented definite symptoms of poliomyelitis on intracerebral inoculation, were found also to neutralize completely a small, though ample dose, approximately 50 M.L.D. of a highly active virus filtrate of the pooled mixed virus strain. The results of the neutralization tests of sera 12 and 16 are especially significant. Although the monkeys from which they had come had proved ordinarily susceptible to intracerebral

TABLE III.

Results on Reinoculation.

Monkey	Method of immunization	Immunization ended	Intracerebral test			Result	
			Date	Amount	Strain	Test animal	Control
First intracerebral inoculation							
2	Intradermal M.A.	1/19/28	6/11/28	0.3 cc. 5 per cent suspension	Aycock	No symptoms	Prostrate on 30th day
9	Subcutaneous M.A.	1/27/28	6/11/28	0.3 cc. 5 per cent suspension	Aycock	No symptoms	
10	Subcutaneous M.A.	1/27/28	6/11/28	0.3 cc. 5 per cent suspension	Aycock	No symptoms	
Second intracerebral inoculation							
2	Intradermal M.A.	1/19/28	12/ 5/28	0.2 cc. 5 per cent suspension	Pooled mixed virus	No symptoms	Prostrate on 7th day
9	Subcutaneous M.A.	1/27/28	12/ 5/28	0.2 cc. 5 per cent suspension	Pooled mixed virus	Prostrate on 12th day	
10	Subcutaneous M.A.	1/27/28	12/ 5/28	0.2 cc. 5 per cent suspension	Pooled mixed virus	No symptoms	

TABLE IV.

Serum Neutralizations.

No.	Treatment		Test		Result	Neutralization				Result	
	Dose	Virus	Date	Virus		Virus	Virus treatment	Route	Amount serum	Test	Control
					Intradermal						
1	42	M.A.	Died intercurrent infection			*cc.* 0.3 M.A. Pooled	2 hrs. incubator. Overnight icebox	Icer.	*cc.* 0.9	No symptoms	Typical polio
2	66	M.A.	1/19/28	M.A.	No symptoms						
3	48	M.A.	1/19/28	M.A.	Slight excitement						
4	42	M.A.	5/31/28	Aycock	Slow	0.1 M.V.		Icer.	0.9	No symptoms	Typical polio
5	42	M.A.	5/31/28	Aycock	Tremor, ataxia, weak deltoid	0.1 M.V.		Icer.	0.9	No symptoms	Typical polio
6	42	M.A.	5/31/28	Aycock	Slow	0.1 M.A.		Icer.	0.9	No symptoms	Typical polio
7	56	Aycock	11/20/28	M.A.	Slow and excited	0.3 M.A.		Icer.	0.9	No symptoms	Typical polio
8	56	Aycock	11/20/28	M.A.	Excitement and tremor	0.2 M.V.		Cist.	0.8	No symptoms	Typical polio

Subcutaneous

9	45	M.A.	2/11/28	M.A. 0.5	No symptoms	0.3 Pooled	2 hrs. incubator. Overnight icebox	Icer.	0.9	No symptoms	Typical polio
10	42	M.A.	2/11/28	M.A. 0.5	No symptoms						
11	38	M.A.	2/11/28	M.A. 0.5	No symptoms						
12	42	M.A.	5/31/28	Aycock 0.5	Prostrate	0.1 M.V.		Icer.	0.9	No symptoms	
13	42	M.A.	5/31/28	Aycock 0.5	Paralyzed	0.1 M.V.		Icer.	0.9	Died intercurrent disease. Serum contaminated	
14	42	M.A.	5/31/28	Aycock 0.5	Prostrate	0.2 M.V.		Cist.	0.8	Late polio	Typical polio
15	56	Aycock	11/20	M.A. 0.5	No symptoms	Not done					
16	56	Aycock	11/20	M.A. 0.5	Paralyzed	0.2 M.V.		Cist.	0.8	No symptoms	Typical polio

inoculation of the active pooled virus, their sera neutralized the same potent material. It is interesting to note that in one instance of the 16 animals tested did the serum fail to exhibit neutralizing power.

The results of the serum neutralization tests show that degrees of immunity to poliomyelitis virus exist not only in monkeys but suggest that the same condition exists in man. The employment of relatively small doses of filtrate of a virus strain, whose potency is quite constant, brings out degrees of specific protection in monkeys. Such variations would have been totally obscured by the ordinary means of determining immunity by the intracerebral inoculation of considerable amounts of suspension of virus possessing varying degrees of infective power. It is conceivable that the past failures of certain efforts to induce immunity in monkeys may be explained, not by the inadequacy of the methods employed, but rather by the overwhelming inoculation which the animal was required to withstand, doubtless far greater than that to which any human would be exposed. The intracerebral test inoculation particularly, with its associated damage to nervous tissue, makes demands upon the immune reaction of an animal many times greater than that arising in any natural method of infection.

CONCLUSIONS.

1. The introduction of considerable amounts of living, active poliomyelitis virus into the skin and subcutaneous tissue of monkeys protects the animals against intracerebral inoculations of similar virus material.

2. The degree of protection conferred by intradermal is greater than by subcutaneous injection.

3. During intradermal and subcutaneous inoculations, no local or general pathological signs were observed.

4. The degree of protection produced by the immunization methods used is not absolute, since a percentage of the inoculated monkeys respond to intracerebral injections of highly potent virus.

5. The sera of the animals inoculated intradermally or subcutaneously neutralized poliomyelitis virus *in vitro*, irrespective of the result of intracerebral inoculation, in all except one instance.

6. The power of the serum of treated monkeys to neutralize virus

in vitro is a more delicate test of immunity than is the intracerebral inoculation.

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STUDIES ON SOUTH AMERICAN YELLOW FEVER.*

I. The Strains of Virus in Use at the Yellow Fever Laboratory in Bahia, Brazil.

By N. C. DAVIS, M.D., and A. W. BURKE, M.D.

(*From the International Health Division of the Rockefeller Foundation, New York.*)

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Up to the time of writing, the middle of January, 1929, there have been five strains of yellow fever virus studied in *Macacus rhesus* monkeys at the laboratory in Bahia. One of these strains came from Rio de Janeiro, three were picked up from local cases of yellow fever, and one was first established in Africa.

In order that references to these viruses in future publications may be more comprehensible, it is our purpose to state briefly how we came into possession of the strains.

F. W. Strain.—This strain was received from Rio de Janeiro through the kindness of Dr. Aragão. The human patient, F. W., was a Hungarian, male, 16 years old. He proved to have a mild attack of the disease. Blood was taken approximately 48 hours after onset. The virus had gone through four passages before being given to us.

Early in September, 1928, Dr. J. H. Bauer brought to Bahia blood and tissues from fifth and sixth passage monkeys which he had inoculated in Rio de Janeiro. He also brought mosquitoes which had fed on these monkeys. Among the animals inoculated in Bahia after his arrival, two showed temperature curves suspiciously like those of mild yellow fever. However, direct transfers from these animals were negative; mosquitoes fed on one were definitely proved non-infective and those fed on the second were doubtfully infective.

Previous to Dr. Bauer's return to Bahia, blood and tissues had been received by airplane. At the time it was thought that the results of inoculating this material were negative. One animal showed a temperature of 104.8° on the fourth day, but after lancing a superficial abscess at the point of inoculation the fever dropped. However, at the height of the fever a batch (No. 6) of *Aedes ægypti* was fed on

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TABLE 1.

Fatal Infections of Monkeys after Inoculations with Brazilian Yellow Fever Virus.

Strain	*M. rhesus* No.	Temperatures, a.m. and p.m.											Remarks
		Day of inocul.	1	2	3	4	5	6	7	8	9	10	
B. B.	6	—	103.8	103.0	103.8	104.9	101.9						Previously used animal. Inoculated with 3 cc. liver emulsion from *rhesus* No. 180
		103.2	103.4	103.6	102.3	105.3	Dead						
"	8	103.4	103.6	102.6	104.4								Previously used animal. Inoculated with 4 cc. blood mixture from *rhesus* Nos. 171, 173, 180
		103.7	102.2	103.8	101.9	Dead							
"	12	—	103.1	102.0	102.9	103.8							Previously used animal. Inoculated with 3 cc. liver emulsion from *rhesus* No. 180
		102.9	103.2	102.5	104.7	104.0	Dead						
"	14	103.0	103.1	103.7	103.0								Previously used animal. Inoculated with 4 cc. blood mixture from *rhesus* Nos. 171, 173, 180. No fever, but autopsy lesions typical
		103.4	102.6	103.9	102.3	Dead							
"	23	103.4	102.9	103.2	102.6								Previously used animal. Inoculated with 4 cc. blood mixture from *rhesus* Nos. 171, 173, 180. No fever, but autopsy lesions typical
		103.6	103.0	103.2	102.6	Dead							
"	24	—	102.4	102.7	103.9	96.8							Previously used animal. Inoculated with 3 cc. blood-liver mixture from *rhesus* Nos. 86, 188, 192. Sacrificed when moribund
		103.0	101.9	102.4	105.1	Dead							
"	47	—	104.4	103.9	104.9								Previously used animal. Inoculated with 3 cc. blood-liver mixture from *rhesus* Nos. 86, 188, 192. Sacrificed when moribund
		102.9	104.3	103.3	103.8	Dead							
"	51	—	103.7	103.4	103.9	103.9	95.7						Previously used animal. Inoculated with 3 cc. blood-liver mixture from *rhesus* No. 141. Sacrificed when moribund
		103.4	103.2	102.4	104.0	104.3	Dead						
"	72	—	103.0	104.7	105.0	103.0							Previously used animal. Inoculated with 2.5 cc. citrated blood from *rhesus* No. 166. Sacrificed on evening of 4th day, when moribund
		103.7	103.2	105.0	104.5	98.5							

"	86	—	102.9	103.4	105.0	103.6							Previously used animal. Inoculated with 2.5 cc. citrated blood from *rhesus* No. 166. Sacrificed on evening of 4th day, when moribund
		103.6	103.1	104.4	104.4	94.2							
"	111	—	103.6	103.5	103.2	103.0	105.0	104.8	104.5	101.7			New animal. Inoculated with 5 cc. citrated blood from *rhesus* No. 107. Sacrificed when moribund
		104.9	103.5	103.7	103.4	103.6	105.2	104.6	104.9	95.0			
"	124	—	103.4	103.8	103.4	103.6	104.5	102.9	102.0				New animal. Inoculated 3 times with a total of 20 cc. from 4 animals
		104.7	103.7	104.4	104.4	104.6	103.4	102.6	102.2	Dead			
"	126	—	102.9	103.8	104.6	106.2	99.6						New animal. Inoculated with 8 cc. citrated blood from 4 animals. Sacrificed when moribund
		—	103.9	104.8	104.9	105.6	98.4						
"	128	—	102.1	104.0	102.3	102.4	104.7	102.8					New animal. Inoculated with 9 cc. citrated blood mixture from *rhesus* Nos. 124 and 125
		102.9	103.8	102.1	105.3	104.8	105.4	Dead					
S. R.	133	102.8	104.4	103.9	103.6	104.4	93.0						New animal. Inoculated with 7 cc. blood from *rhesus* No. 116 (1st passage from S. R.). Sacrificed when moribund
		103.8	103.9	103.0	103.9	106.0							
B. B.	136	—	102.9	103.2	104.5	104.9	101.2						New animal. Inoculated with 4 cc. citrated blood from *rhesus* No. 128
		103.7	102.8	103.5	103.9	105.0	Dead						
"	144	—	103.9	103.4	103.8	103.9							New animal. Inoculated with 8 cc. liver emulsion from *rhesus* Nos. 136 and 137
		103.9	103.7	103.9	103.5	105.3	Dead						
"	155	—	103.6	104.6	105.3	105.0	102.9	102.9					New animal. Inoculated with 8 cc. blood from *rhesus* No. 144. Autopsy showed tuberculosis also
		103.1	103.4	104.2	106.0	104.2	103.9	102.0	Dead				
"	158	102.0	102.8	102.9	103.4	102.8	102.3						Previously used aninal. Inoculated with 3 cc. blood-liver mixture from *rhesus* Nos. 24, 47, 197
		102.5	102.7	103.0	102.7	105.3	102.7	Dead					
"	170	103.9	103.2	104.0	104.2	104.5	104.6						New animal. Inoculated with 10 cc. liver emulsion from *rhesus* No. 161. Liver frozen 10 days. Animal sacrificed when moribund on 5th day
		103.4	103.0	104.4	105.0	106.4	97.2						

Note: Animals 111 to 155, inclusive, were infected earlier chronologically than animals 6 to 86; apparently, the virus was more active when used in the later experiments.

Animals infected through mosquito transmission will be considered in a separate paper.

TABLE I.—*Continued.*

Strain	*M. rhesus* No.	Temperatures, a.m. and p.m.											Remarks
		Day of inocul.	1	2	3	4	5	6	7	8	9	10	
B. B.	180	—	103.6	105.4	104.6								New animal. Inoculated with 2.5 cc. citrated blood from *rhesus* No. 166. Sacrificed. Not moribund, but would not have lived until following day
		102.9	103.8	104.5	102.4								
"	188	103.6	102.9	103.4	104.2	103.9							New animal. Inoculated with 4 cc. blood mixture from *rhesus* Nos. 171, 173, 180
		102.7	102.5	104.5	105.3	103.6	Dead						
"	192	—	102.8	102.6	104.6	105.4							New animal. Inoculated with 2 cc. blood-liver mixture from *rhesus* Nos. 170, 180
		102.4	103.8	102.2	106.2	105.6	Dead						

this monkey. These mosquitoes, together with batch No. 7, fed on a sixth passage animal in Rio de Janeiro, were allowed to feed on *rhesus* No. 52 on Sept. 17. The following day the temperature of this animal reached 106.1°F. and it passed 104°F. daily for five days. On the seventh day the monkey was killed. The liver showed some fat and a thin scattering of necrotic cells. It was evident that the animal was on the road to recovery when sacrificed.

From mosquitoes fed on *rhesus* No. 52 the strain was maintained. Separate feeding and separate injection of mosquito emulsion of batches Nos. 6 and 7 caused no rise of temperature in experimental animals. However, later tests indicated that *both* batches had caused immunity.

The F. W. strain in our hands has not proved very virulent. It sometimes gives rise to a rather severe temperature reaction, but it has caused death in only three instances.

B. B. Strain.—On Sept. 2, 1928, the writers were notified of a suspected case of yellow fever in the person of B. B., a Russian Jew, 16 years of age, who had been one year in Brazil. Blood was drawn at 10.30 a.m. and inoculated into two guinea-pigs and a *rhesus* monkey. The boy was said to have been taken sick at 10 a.m. on Aug. 30, although there was an indefinite history of indisposition since the evening of the 29th. At the time of our visit he lay hunched up in bed, rather dull, with pulse of 90 and temperature of about 103°F. The eyes were congested and slightly icteric. The tongue was pointed, had red margins, and was furred white on the dorsum. The lips were cracked and excoriated. He had complained of headache previously. There had been vomiting, but no black vomit. Urine was said to contain casts and 1 gm. of albumen per liter. Later he developed bleeding gums, and black vomitus appeared. It was clinically a frank case of yellow fever. On Sept. 4 he became semicomatose and died in the evening. No autopsy was obtainable.

On the seventh day after inoculation one guinea-pig developed a fever of 105.2°, but transfer to another animal was negative and blood cultures in semi-solid medium gave no growth. On the eleventh day the *rhesus* monkey (No. 31) had a temperature of 104.3° and transfer was made to another animal (No. 45). The latter showed fever on the seventh day. Third and fourth passage animals both had high fever on the third day and were sacrificed to obtain tissues for Dr. Bauer to take to New York. A fifth passage animal (No. 63) died, but with a complicating peritonitis. After the seventh passage direct transfers lost in virulence for a time, owing, probably, to shortage of animals and the necessity of using partially immune monkeys. The strain was established again in virulent form through mosquitoes. The batch fed on *rhesus* No. 63 caused a fatal infection when allowed to feed on *rhesus* No. 98. From this point the virus has been carried by a combination of direct transfers and mosquito transmission through a total of at least 14 passages. To the end of December, 1928, 135 animals had been inoculated with this strain, of which 26 had either died or been sacrificed when moribund. However, this small percentage of deaths does not give a true picture of the present virulence, since the total (135) includes all those animals used in building up the virulence, many

negative or partially successful mosquito transmissions, and a large number of animals inoculated with other material and later tested for immunity with the B. B. virus. Autopsies on fatal cases have shown the classical picture of yellow fever. (See reports by Hudson on African studies.[1])

S. R. Strain.—On Oct. 26, 1928, Dr. Eduardo de Araujo notified us that he had heard of a suspicious case of fever. However, the attending physician had not considered it sufficiently suspicious to report to the Health Department.

We found the patient, S. R., to be a Spanish woman, married, 18 years of age. She had become ill on the afternoon of Oct. 23, *i.e.*, approximately 66 hours before our visit. Fever had risen to 104°F., but was down to 100.8°, axillary. The eyes were not injected nor icteric. The tongue was slightly coated, but was not pointed, nor did it have the red margin frequently seen in yellow fever. There had been a little bilious vomiting, but no black vomit. The attending physician reported the urine to have albumen in considerable amount. The patient appeared to be in no pain, was bright and attentive, but not anxious. On the fifth day she was frankly convalescent, with normal pulse and temperature, without ever having shown jaundice or black vomit. The only suspicious signs had been the albumen in the urine and a rather high temperature.

Blood taken at the time of our visit (66 hours after onset) was inoculated into two guinea-pigs and two *rhesus* monkeys (Nos. 116 and 117). On the seventh day *rhesus* No. 116 showed a rise of temperature to 104.2°F. and blood transfer was made to *rhesus* No. 133. The latter had a fever on the day after inoculation but not again until the fourth day, when the temperature rose to 106.2°F. The animal died on the fifth day with typical gross and microscopic lesions of yellow fever. Mosquito batch No. 53 was allowed to feed on this monkey on both the first and fourth days. Blood transfer from the same monkey to *rhesus* No. 142 was made on the fourth day, and liver from the autopsy was emulsified and inoculated into *rhesus* No. 143. The two animals inoculated had no obvious reaction. On Dec. 1 only two stegomyias remained alive in batch No. 53. These engorged on *rhesus* No. 168 and the latter developed a fever of 104.4° on the fourth day, but recovered. Blood from this monkey (No. 168) was injected into *M. rhesus* No. 186, which died from other causes before showing any signs of yellow fever. The surviving two mosquitoes of batch No. 53, together with seventeen others whose remains were fished from the wet cotton and the pan of honey in the cage, were emulsified and inoculated into *rhesus* No. 177, without result. Mosquito batch No. 72 which had engorged on *rhesus* No. 168 was allowed to feed on *rhesus* No. 217. On the next day the temperature of this animal reached 104.0° and in the second day rose to 104.8°. Blood transfer to *rhesus* No. 228 proved positive.

The following animals which received S. R. virus have been proved immune to B. B. strain virus, having given no temperature reaction following the inoculation

[1] Hudson, N. Paul, *Am. J. Path.*, 1928, iv, 395.

of virulent material: No. 117 (one of the original blood inoculations), No. 143 (inoculated with emulsified liver), No. 168 (fed upon by mosquitoes of batch No. 53) and No. 177 (inoculated with emulsified mosquitoes of batch No. 53). *M. rhesus* No. 116, of the first passage of the S. R. strain, proved absolutely resistant to the African strain of virus.

J. V. O. Strain.—On Sept. 14, 1928, Dr. Barros Barreto came to tell us that a suspected case had been admitted to the Isolation Hospital. Later in the evening the patient, J. V. O., a young Spaniard, was seen by Dr. Bauer and the writers. Although only at the end of the third day of the disease, he was already comatose and had a normal to subnormal temperature. There was noted muscular twitching and bleeding from nose and rectum. It was considered useless to take blood for inoculation. He died at 5 a.m., Sept. 15, and the autopsy was started at about 9:30 a.m. by Dr. Eduardo de Araujo. Icterus was very marked in the dead body. Autopsy findings were typical of yellow fever, including sub-epicardial hemorrhages, bleeding into stomach and intestines, box-wood liver, and intensely injured kidneys.

Liver, spleen, and kidney tissues taken at autopsy were emulsified separately and three animals were inoculated. *Rhesus* No. 48 received spleen emulsion. A severe necrosis of the abdominal wall developed and the monkey was sacrificed on the fifth day. There was no evidence of yellow fever. *Rhesus* No. 47 received liver emulsion. A small abscess appeared and there was a slight fever for two days. Blood transfer to a normal animal gave no results, and No. 47 recovered. On Dec. 10 this monkey was inoculated with virulent B. B. strain material and died on the fourth day with typical yellow fever. No immunity had developed.

M. rhesus No. 46 received kidney emulsion. Here also an abscess resulted. For four days following injection there was a fever, which upon one occasion reached 105.6°. On the second day blood transfer was made to *rhesus* No. 22, but culture of this blood made by Dr. Bauer, yielded a gram negative organism thought to be *B. coli*. *M. rhesus* No. 22 reacted with a high fever, but no further transfers were made, because we thought that the infection was bacterial; the animal recovered. The temperature of *rhesus* No. 46 again reached or passed 104°F. on the afternoons of the twelfth, fourteenth, and sixteenth days after inoculation. Suddenly on the morning of the twentieth day the temperature went up to 105°F. and the monkey was sacrificed. Blood culture at this time yielded a pure growth of streptococci. Both blood and liver were transferred to *rhesus* No. 44. The latter animal had previously been used for experimentation but was considered to be non-immune. Sections of the liver of *rhesus* No. 46 showed the deposition of considerable fat, but apparently no necrosis.

M. rhesus No. 44 developed an abscess at the point of inoculation and the temperature passed 104°F. on the first and third days. On the fourth day there was a rise to 105.9° and mosquito batch No. 29 was allowed to feed. No blood transfer was made because it was thought that the fever arose from bacterial infection. *M. rhesus* No. 44 proceeded to recover, with only one more marked rise in temper-

ature on the tenth day. On Dec. 8, *rhesus* No. 44 was inoculated subcutaneously with 3 cc. of liver emulsion from *rhesus* No. 180, B. B. strain. Not the slightest temperature reaction resulted, although control *rhesus* No. 188 died with typical yellow fever on the fifth day.

On Nov. 1 mosquito batch No. 29, which had fed on *rhesus* No. 44, was allowed to feed on *rhesus* No. 132. Four days later the whole batch (58 remaining mosquitoes) was killed, ground up, and inoculated into the same animal. On Nov. 11 there appeared a fever of 104.6°F. and blood transfer was made to *rhesus* No. 154. The latter had a fever on the second and third days. Blood transfer was made to *rhesus* No. 158, and mosquito batch No. 61 was fed. *M. rhesus* No. 154 later developed a more or less continuous fever and it was sacrificed; early tuberculosis was present. *M. rhesus* No. 158 had a slight fever, beginning on the seventeenth day after inoculation and appearing on three days. However, this was apparently not yellow fever, because ten days after the last febrile access the animal was inoculated with B. B. strain virus and succumbed on the sixth day.

On Dec. 1 mosquito batch No. 61, which had fed on *rhesus* No. 154, was allowed to feed on *rhesus* No. 174, and this monkey had a fever of 104°F. on the second day. Eleven days after the infective feed, virulent B. B. strain material (the same as used for No. 158) was inoculated but gave no reaction. This J. V. O. strain, carried in mosquitoes, has been dropped, since it appeared to be avirulent and of no use for experimental purpose.

On Sept. 15, the day of the death of J. V. O., the writers and their assistants captured 59 female *Aedes ægypti* in the bedroom where the patient had slept during his illness until removed to the Isolation Hospital. Upon three occasions, the first time eight days after capture, these mosquitoes were allowed to feed upon *rhesus* No. 60. Five days after the first feeding and three days after the second, this monkey's temperature reached 103.9°F. There was then a drop until two days later when 104.4° was reached. Blood transferred at this point gave no reaction. The mosquitoes which fed on No. 60 were allowed to engorge on one normal monkey without result, but were destroyed through an error before further experimentation could be carried out. The original mosquitoes caught in the bedroom of J. V. O. were eventually ground up and inoculated into two monkeys. These showed no fever and were later proved susceptible to B. B. strain virus. Evidently the virus, if present, was not sufficient either to infect or to immunize two animals. However, *rhesus* No. 60, which had shown a temperature reaction following bites, appeared to be resistant upon further inoculation.

African Strain.—This is the Asibi strain, which has been in use for many months at the laboratory of the West African Yellow Fever Commission of the Rockefeller Foundation in Lagos, Nigeria. It was later established by Dr. Sawyer in New York, sent from there to Rio de Janeiro in November, 1928, established at the Oswaldo Cruz Institute, and sent from Rio to us through the kindness of Dr. Aragão. In our hands it has gone through five passages and has killed eleven monkeys.

TABLE II.

Fatal Infections of Monkeys after Inoculation with African Yellow Fever Virus.

Strain	*M. rhesus* No.	Temperatures, a.m. and p.m.											Remarks
		Day of inocul.	1	2	3	4	5	6	7	8	9	10	
Asibi	175	—	104.7	103.9	103.1	106.5	104.9						Inoculated with monkey liver sent from Rio de Janeiro. Developed abscess
		103.4	104.1	103.9	103.9	106.0	103.3	Dead					
"	185	—	103.7	106.0									Inoculated with 2 cc. blood from No. 175 (taken on 4th day)
		103.9	104.9	104.0	Dead								
"	190	—	102.7	102.9	103.6								Inoculated with 2.5 cc. citrated blood from No. 185. Sacrificed when moribund on evening of 3rd day
		102.7	102.2	104.6	100.4								
"	118	—	102.0	103.9	103.4	104.0	104.2	101.7					Previously used animal probably partially immune. Inoculated with 2.5 cc. citrated blood from No. 185
		101.8	102.3	103.7	103.2	103.6	105.0	102.0	Dead				
"	201	—	103.1	103.9	104.4								Inoculated with 2 cc. blood-liver mixture from No. 190
		103.8	103.6	105.1	104.4	Dead							
"	216	102.9	103.0	103.6	102.9	103.8	103.9	99.1					Inoculated with 10 cc. blood-liver mixture from No. 201. Blood 19 days old, liver 17 days old (frozen most of time). Sacrificed when moribund
		102.7	103.4	103.5	103.0	103.6	104.8						
"	229	—	102.7	104.4	105.0	102.4							Inoculated with 2 cc. citrated blood from No. 209 (the latter infected by mosquitoes). Sacrificed when moribund
		103.0	103.8	105.7	104.9								
"	204	103.1	103.5	103.4	104.2	104.0	99.0						Previously used animal. Inoculated with 2 cc. citrated blood from No. 209
		103.3	103.8	103.6	104.4	104.2	Dead						
"	189	—	102.5	103.6	104.8	104.6	105.8	102.0					Previously used animal. Inoculated with 2 cc. citrated blood from No. 209
		102.0	103.1	103.8	105.4	104.8	105.2	Dead					

DISCUSSION AND CONCLUSION.

It would appear that inoculation of *rhesus* monkeys served a diagnostic purpose in one of our cases, that of S. R. The disease was so mild that in the absence of an epidemic no clinician would have made the diagnosis without this laboratory procedure.

It should be pointed out that following inoculation of liver emulsion, and sometimes even of blood, it is impossible to tell whether an early fever resulting is due to a protein reaction, to bacterial infection, or to true yellow fever. If plenty of animals are available the only safe plan is to take blood for subinoculation and for cultures, and to feed mosquitoes. Blood may be kept frozen for some time before inoculation if it is found to be necessary, or if it is desired to await the outcome of the disease in the animal bled.

A combined blood and liver transfer showed the presence of virus in *rhesus* No. 46 twenty days after the original inoculation with kidney from the autopsy in the case of J. V. O. The same animal had had an infected body wall and a bacteriemia.

On the basis of later experience we can see some of our mistakes. In the second S. R. passage (*rhesus* No. 133) blood transfer should have been made at the time of initial fever, the day following inoculation. In the case of *rhesus* No. 60, mosquito infection from J. V. O,, transfer probably should have been made when the temperature reached 103.9°F. on the fifth day. Two days later, with a fever of 104.4°F., the blood appeared to be non-infective. A temperature of 104°F. in monkeys is usually a safe borderline between fever and no fever, but seemingly not always.

Our experience with the South American viruses so far indicates that it takes much care, patience, and an abundance of monkeys to build up and maintain a high degree of virulence.

For invaluable help in securing these yellow fever strains, and for much assistance and advice in establishing the laboratory we gratefully acknowledge our indebtedness to Dr. J. H. Bauer of the West African Yellow Fever Commission of the Rockefeller Foundation, to Dr. Henrique de Beaurepaire Aragão, of the Oswaldo Cruz Institute, Rio de Janeiro, to Dr. Antonio Luiz C. A. de Barros Barreto, Director of the Department of Health, State of Bahia, and to Dr. Eduardo de Araujo, Director of the Oswaldo Cruz Institute, São Salvador, State of Bahia.

STUDIES ON SOUTH AMERICAN YELLOW FEVER.*

II. Immunity of Recovered Monkeys to African Virus.

By NELSON C. DAVIS, M.D.

(*From the International Health Division of the Rockefeller Foundation, New York.*)

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The purpose of the present paper is to offer further proof of the identity of Brazilian and African yellow fever viruses. In addition to the clinical and pathological evidence, the literature at the present writing contains two reports indicating immunological identity of the strains. In June, 1928, Dr. Aragão (1) received African virus, brought from Paris by Dr. Carlos Chagas. This was apparently of low virulence and caused only a mild febrile reaction. However, the two inoculated animals later proved immune to a Brazilian strain. Recently a valuable paper by Theiler and Sellards (2) has presented the results of protection experiments in which immune sera from human cases of the Parahyba, Brazil, outbreak of 1926 were tested against African virus. Some of the sera were not potent, but others gave absolute protection.

On Dec. 1, 1928, we received African virus, Asibi strain, from the Oswaldo Cruz Institute in Rio de Janeiro, through the kindness of Dr. Aragão. The strain had previously been established in New York by Dr. Sawyer and was sent by him to Rio de Janeiro, arriving there early in November. The establishment of this virus in Bahia has enabled us to test the immunity of a number of animals recovered from Brazilian yellow fever.

The results are presented in Table I. In addition to four non-immune controls, three of which promptly died, twenty-two supposedly immune animals were inoculated. Of the twenty-two one died of typical yellow fever, although death occurred three days later than

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation.

TABLE I.

Results of Inoculation of African Virus in Monkeys Recovered from Brazilian Yellow Fever.

Rhesus No.	Original Brazilian strain	Temperatures Recorded, a.m. and p.m.																				
		Day of inoculation	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Experiment I. Inoculated with 2.5 cc. citrated blood subcutaneously from *rhesus* No. 185, African strain, on Dec. 7																						
190	Control	—	102.7	102.9	103.6	Sacrificed when *in extremis*, evening of 3rd day. Yellow Fever																
		102.7	102.2	104.6	100.4																	
116	S. R.	—	102.2	102.3	101.9	102.2	102.7	102.6	102.6	102.4	102.2	102.1	102.6	102.0	102.2	102.0	102.0	102.1	102.3	102.2	102.0	102.4
		103.0	102.7	102.0	102.0	102.4	102.8	102.6	102.9	102.4	102.6	102.4	102.4	102.7	102.5	102.1	102.5	—	102.5	—	102.2	—
118	B. B.	—	102.0	103.9	103.4	104.0	104.2	101.7	Dead	Yellow Fever												
		101.8	102.3	103.7	103.2	103.6	105.0	102.0														
122	F. W.	—	104.0	102.6	102.1	101.7	102.1	102.0	102.4	103.6	103.8	102.9	103.2	103.1	103.0	103.1	102.0	102.6	102.5	102.4	102.6	102.4
		104.8	104.0	102.0	102.8	102.0	101.9	101.3	103.9	104.9	103.8	103.0	103.4	103.3	103.2	103.2	102.8	—	102.7	—	102.8	—
125	B. B.	—	102.6	103.0	103.4	102.2	102.6	101.9	102.0	101.5	102.4	102.1	102.0	101.9	102.0	102.1	102.2	102.2	102.6	102.2	102.3	102.5
		103.0	102.0	103.6	104.0	102.0	101.7	101.5	101.9	102.0	102.2	101.9	101.7	102.1	101.9	102.4	102.4	—	102.4	—	102.6	—
31	B. B.	—	102.7	102.4	102.8	102.5	102.0	102.3	102.6	102.9	103.2	103.4	103.2	102.9	102.8	103.2	101.6	103.2	103.1	102.9	102.6	103.5
		103.0	103.0	102.6	103.0	101.8	101.6	101.7	102.8	103.0	103.6	103.4	103.0	103.1	103.0	103.4	103.0	—	103.4	—	102.2	—
Experiment II. Inoculated 2 cc. blood-liver mixture subcutaneously from *rhesus* No. 190, African strain, on Dec. 12																						
201	Control	—	103.1	103.9	104.4	Dead	Yellow Fever															
		103.8	103.6	105.1	104.4																	
167	F. W.	102.9	103.6	103.0	103.1	103.4	102.4	103.0	103.4	103.0	103.6	102.6	102.9	102.7	102.9	103.6	103.9	103.9	104.0	103.7	103.4	103.8
		103.8	103.3	103.1	103.3	102.9	102.9	103.1	103.2	103.5	103.6	102.8	—	103.4	—	103.8	104.0	104.0	103.9	103.9	103.6	—
50	B. B.	—	102.2	103.0	102.6	102.8	103.2	103.4	102.9	103.1	103.3	102.0	101.9	102.0	102.8	102.5	102.7	103.0	103.6	102.9	102.7	
		102.0	102.5	102.9	102.2	103.0	103.5	103.5	103.0	103.2	103.6	102.0	—	102.2	—	102.4	102.9	103.4	102.5	—	—	
65	B. B.	—	102.8	103.6	103.0	102.9	103.1	102.9	103.1	102.9	103.4	102.4	102.8	102.6	102.1	102.3	102.2	102.8	103.0	102.8	103.0	
		103.2	103.1	103.2	103.6	103.4	102.9	103.0	102.9	103.2	103.3	102.2	—	102.8	—	102.4	102.5	102.9	102.2	—	—	
70	B. B.	—	102.0	103.0	103.0	102.5	102.8	102.4	103.0	102.9	103.0	102.3	103.4	103.1	103.0	102.9	103.3	103.4	102.8	102.5	102.8	
		102.8	102.2	103.4	102.9	102.5	102.6	102.8	103.4	102.9	103.3	103.1	—	103.2	—	103.0	103.5	103.0	102.2	—	—	
121	B. B.	—	102.9	102.8	102.9	103.2	103.0	102.7	103.1	102.4	102.1	102.2	103.0	103.1	103.2	102.8	102.6	102.4	102.6	102.8	102.5	
		102.6	102.2	102.8	103.0	103.4	102.9	103.0	102.6	102.5	103.0	103.2	—	103.4	—	102.9	102.8	102.5	102.6	—	—	

114	B. B.	—	102.7	102.4	103.3	103.1	99.0	Sacrificed when *in extremis*. Dysentery																		
		102.2	104.0	102.9	103.6	103.3	98.4																			
108	B. B.	—	102.7	102.2	102.5	102.4	101.7	102.0	102.8	103.0	103.2	102.1	101.9	102.6	102.0	101.7	102.1	102.0	102.7	103.0	102.9					
		103.3	102.2	102.1	102.8	102.0	101.6	102.4	102.9	103.1	103.2	102.0	—	102.2	—	101.9	102.2	102.3	102.9	—	—					
130	F. W.	—	102.9	102.5	101.4	102.0	101.9	102.7	102.1	102.3	102.7	102.0	101.9	101.7	102.0	101.9	102.2	102.2	102.2	103.0	102.8					
		102.3	101.5	102.0	101.9	102.1	102.0	102.4	101.8	103.1	102.6	102.0	—	101.9	—	102.0	102.4	102.0	102.9	—	—					
131	B. B.	—	102.6	102.7	102.9	102.4	102.4	102.6	102.0	102.4	102.1	101.9	101.7	101.9	101.2	102.0	102.1	102.6	102.8	102.4	102.1					
		102.7	102.9	103.0	102.6	102.6	102.7	102.2	102.4	102.6	102.0	103.4	—	102.0	—	102.2	102.4	102.6	102.3	—	—					
135	F. W.	—	102.6	102.0	102.3	103.6	102.9	103.1	102.9	102.2	102.9	103.1	102.8	102.7	102.4	103.0	103.8	103.7	103.8	103.7	103.6					
		102.0	102.2	102.7	103.0	103.1	103.4	103.5	103.0	103.1	103.0	102.6	—	102.7	—	103.4	103.6	103.9	103.9	—	—					
140	B. B.	—	102.5	102.0	102.8	102.6	102.3	102.8	101.9	102.0	102.1	102.0	101.7	102.0	102.0	102.1	102.3	101.9	102.1	102.4	102.6					
		102.0	102.2	102.6	102.1	102.6	102.5	102.2	101.7	102.3	101.9	102.0	—	102.2	—	102.2	102.0	102.0	102.6	—	—					
147	B. B.	—	102.5	102.6	102.9	103.4	103.0	102.9	103.0	103.4	102.7	102.5	102.0	101.9	102.0	102.3	102.2	102.6	102.7	103.2	103.5					
		101.8	102.0	103.1	103.0	103.4	103.6	102.9	103.3	103.1	102.4	102.1	—	102.1	—	102.4	102.4	102.8	103.0	—	—					

Experiment III. Inoculated 2 cc. citrated blood subcutaneously from *rhesus* No. 209, African strain, on Jan. 5

229	Control	—	102.7	104.4	105.0	102.4	Sacrificed when *in extremis*, afternoon of 4th day. Yellow Fever																			
		103.0	103.8	105.7	104.9	—																				
38	F. W.	—	103.2	104.0	103.9	103.6	103.8	104.6	103.6	103.1	103.3	103.7	103.0	103.6	103.0	103.4	103.0	102.9	103.5	103.7	103.3	103.0				
		102.9	103.4	104.1	104.0	104.1	103.9	104.3	103.4	103.5	103.5	103.1	103.4	103.2	103.6	103.2	—	103.2	103.5	103.4	103.5	—				
49	B. B.	—	103.0	103.5	103.2	103.4	103.7	103.7	102.9	103.6	103.0	103.6	103.4	103.0	102.5	102.4	102.8	102.5	103.0	102.8	102.0	102.0				
		103.4	103.2	103.4	103.3	103.4	103.5	103.6	103.4	103.2	103.4	103.2	103.1	102.9	102.7	102.6	—	102.2	102.9	102.3	101.9	—				
191	B. B.	—	102.5	102.4	103.0	103.0	104.0	103.9	103.7	103.2	103.6	103.6	103.4	103.8	103.4	103.2	102.9	102.7	103.9	103.9	103.8	103.7				
		103.0	102.6	102.0	103.6	103.9	104.4	104.0	103.6	103.4	103.7	103.2	103.2	103.6	103.6	103.0	—	102.6	104.0	103.6	103.5	—				

Experiment IV. Inoculated 2 cc. citrated blood subcutaneously from *rhesus* No. 229, African strain, on Jan. 7

231	Control	—	102.3	102.0	103.3	103.8	104.4	103.0	104.0	103.0	103.4	103.2	103.5	103.6	103.7	103.5	103.0	103.2	103.4	103.5	103.1	103.5				
		102.2	102.5	103.4	103.6	104.8	104.0	104.6	103.9	103.5	103.6	103.1	103.7	103.4	—	103.2	103.4	103.5	103.7	103.3	103.4	—				
44	J. V. O.	—	102.9	103.4	103.5	103.8	103.5	103.6	103.4	102.9	103.5	103.0	103.6	103.4	103.6	103.4	103.2	103.2	103.0	103.3	103.4					
		103.0	103.0	103.1	103.7	103.9	103.8	103.6	103.1	103.3	103.2	103.4	103.5	103.2	—	103.6	103.0	103.5	103.4	102.9	—					
79	B. B.	—	103.0	102.6	102.8	102.9	103.2	103.5	102.9	103.4	102.7	102.8	102.4	102.8	103.4	103.6	103.9	103.9	103.5	103.6	103.7	103.6				
		102.4	102.9	102.7	102.5	103.0	103.3	103.7	103.2	103.0	102.5	102.7	102.6	103.2	—	103.8	104.0	104.2	103.8	103.2	103.8	—				

TABLE II.

Previous History of Monkeys Used in Immunity Tests.

M. rhesus No.	Previous history
	Experiment I
190	Control. Not previously used
116	Inoculated with blood from S. R., Oct. 26. First fever Nov. 2; last fever Nov. 6; highest temperature 104.6°F.
118	Inoculated with blood from *rhesus* No. 104, Oct. 26. First fever Oct. 28; last fever Oct. 31; highest temperature 104.5°
122	Inoculated with blood from *rhesus* No. 100, Oct. 27. First fever Oct. 30; last fever Nov. 7; highest temperature 104.9°
125	Inoculated with blood from *rhesus* No. 118, Oct. 28, and from *rhesus* Nos. 111, 112, 114 and 118 on Oct. 29. First and last fever on Oct. 31; highest temperature 104.7°
31	Inoculated with blood from B. B., Sept. 2. First and last fever on Sept. 13; highest temperature 104.1°
	Experiment II
201	Control. Not previously used
167	Fed upon by mosquito batch No. 38, Dec. 1. First fever Dec. 4; last fever Dec. 6; highest temperature 105.3°
50	Inoculated with blood from *rhesus* No. 64, Oct. 2. First fever Oct. 4; last fever Oct. 7; highest temperature 104.6°. Used as an immune control for injection of tissues of No. 141 in Nov., without reaction
65	Inoculated with blood from *rhesus* No. 49, Sept. 28. First fever Oct. 4; last fever Oct. 5; highest temperature 104.8°
70	Inoculated with blood from *rhesus* No. 64, Oct. 2. First fever Oct. 4; last fever Oct. 14 (2nd rise); highest temperature 104.6°. Used as immune control for injection of blood-liver emulsion from Nos. 51, 152 and 155 in Nov., without reaction
121	Fed upon by mosquito batches Nos. 27 and 30, Oct. 27. First and last fever Nov. 1. Inoculated with blood of Nos. 146 and 129 on Nov. 13; temperature rose to 104.6°, Nov. 19
114	Inoculated with blood from *rhesus* No. 94, Oct. 24. First fever Oct. 28; last fever Oct. 31; highest temperature 105.0°
108	Inoculated with blood from *rhesus* Nos. 79 and 93, Oct. 23. First and last fever on Oct. 30; highest temperature 104.7°
130	Inoculated with blood from *rhesus* No. 123, Nov. 1. First and last fever Nov. 2; highest temperature 104.8°
131	Inoculated with blood-liver emulsion from *rhesus* No. 111, Nov. 1. First fever Nov. 2; last fever Nov. 6; highest temperature 105.4°. Inoculated with blood-liver emulsion from No. 101 in Nov., without reaction

TABLE II—*Concluded.*

M. rhesus No.	Previous history
	Experiment II—*Concluded*
135	Inoculated with blood from *rhesus* No. 130 on Nov. 2 and liver emulsion from No. 123 on Nov. 7. First fever Nov. 9; last fever Nov. 14; highest temperature 104.8°
140	Inoculated with blood from *rhesus* No. 136 on Nov. 5 and 6. First fever Nov. 9; last fever Nov. 10; highest temperature 104.8°
147	Inoculated with blood from *rhesus* Nos. 140 and 141, Nov. 9. First and last fever Nov. 10; highest temperature 104.8°
	Experiment III
229	Control. Not previously used
38	Inoculated with liver emulsion from monkey (series of Dr. Aragão) on Sept. 11. Fever from Sept. 19 to 22 and again from Sept. 30 to Oct. 2. Highest temperature 104.9°
49	Fourth passage monkey of B. B. strain. Inoculated Sept. 23. First and only fever Sept. 28; highest temperature 104.5°
191	Inoculated with blood-liver mixture from *rhesus* Nos. 170 and 180, also with immune serum on same date. Fever on 12th and 14th days; highest temperature 104.2°
	Experiment IV
231	Control. Not previously used
44	Inoculated with blood, liver, and spleen from *rhesus* No. 16, Oct. 5. Fever on Oct. 6, 8, 9 and 15; highest temperature 106.3°. Later immune to B. B. strain
79	Fed upon by mosquitoes Oct. 8. First fever Oct. 23, last fever Oct. 28; highest temperature 104.2°

in the control; another developed dysentery and was sacrificed when *in extremis* on the fifth day; six others showed a mild febrile reaction; fourteen had no fever.

Two of these animals had acquired their infections directly from human blood. Four Brazilian strains were represented. In point of time elapsed since the original attack of fever, one had gone slightly over three months and one only six days, the others for intermediate periods before the immunity test. Five animals had been used as immune controls for B. B. virus between the first attack and the inoculation with African virus.

There is no obvious explanation for the susceptibility of *rhesus* No. 118, which succumbed to the African virus. This animal apparently survived a typical, but only moderately severe, attack of the disease contracted by blood inoculation from *rhesus* No. 104. The latter was infected by mosquito bites and was a fairly severe case; the serum of *rhesus* No. 104 (taken 15 days after recovery) protected normal *rhesus* No. 191 against a large dose of B. B. virus, the control with non-immune serum, No. 192, being dead on the fifth day.

The origin of the Brazilian strains mentioned in Table I has been considered in a preceding paper (3). The African (Asibi) strain reached our hands on Dec. 1, 1928, and the *rhesus* (No. 175) inoculated on that date was found dead on Dec. 7. *M. rhesus* No. 185, whose blood was used in Experiment I of this series, was a subinoculation from No. 175 and also a fatal case. *M. rhesus* No. 190, third passage in our hands, was used for the inoculation of the animals in Experiment II. *M. rhesus* No. 209, the blood of which was used in Experiment III, was also a third passage, since it was infected from mosquitoes which had bitten No. 185. All fresh animals in first to fourth passages, as well as a fifth passage animal (subinoculation from No. 201), died with typical yellow fever. The control in Experiment IV, No. 231, was the first failure encountered. Even in this instance the onset of fever was perfectly definite and there is no doubt in our minds that the animal had yellow fever. The same dosage of virus from No. 229 killed *rhesus* No. 19.

Although Theiler and Sellards (2) are inclined to discount the value of temperature observations, we are disposed to regard then as valuable diagnostic aids when judiciously interpreted. It is perfectly true that one does not always detect a fever in fatal cases, even when temperatures are taken twice daily. It is equally true that many febrile reactions are not yellow fever. However, the indications are so often right that we consider well spent the time needed for this work.

CONCLUSIONS.

These experiments indicate that, although there are quantitative differences in virulence and minor differences in behavior, the African strain and the Brazilian strains of yellow fever virus tested are immunologically the same.

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ETIOLOGY OF OROYA FEVER.

XIV. The Insect Vectors of Carrion's Disease.

By HIDEYO NOGUCHI, M.D., RAYMOND C. SHANNON, EVELYN B. TILDEN, and JOSEPH R. TYLER.

(From The Rockefeller Institute for Medical Research, and the International Health Division of the Rockefeller Foundation, New York.)

Plates 45 to 47.

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It is desirable to present the two parts of this investigation in a single paper, since they bear on each other so closely that to publish them separately will call for considerable repetition of statement. The origin of the studies which have led to the results here presented is to be found in the earlier papers of this series (1), and in the work of Townsend (2), who concerned himself especially with the insect vector of the disease embraced under the names of Oroya fever and verruga peruana. Since the observations given in this paper were completed after the death of Dr. Noguchi, who planned the experiments, we wish to state briefly the circumstances surrounding the investigation.

The earlier papers of the series established *Bartonella bacilliformis* as the bacterial incitant of Oroya fever (Carrion's disease) and verruga peruana. The etiology and much of the pathology of these manifestations of a single infectious disease were made clear by the experimental studies (Noguchi) carried out between 1925 and 1928. The essential fact which remained to be determined was the precise mode of infection in the two maladies for which the history and clinical observation had indicated an insect vector. Townsend's studies of the distribution of the disease and the nocturnal nature of its origin had led him to the decision that the vector belonged to the class of phlebotomi. Indeed, he had gone so far as to designate the vector as *Phlebotomus verrucarum*.

The cooperation of the International Health Division of the Rockefeller Foundation was secured in the field investigation of insects in the verruga zones in Peru. Various insect species were collected by one of us (Shannon), identified as far as possible on the grounds, the identifications being completed afterwards in the United States, and sent by ship to New York, where they were tested for infectivity on monkeys according to Dr. Noguchi's plans (Tilden and Tyler). The procedure employed in this testing was as follows:

The insects were collected without the use of chemicals and sealed alive in sterile tubes, which were either dry, or contained a piece of absorbent cotton moistened with sterile citrate solution (about half of each shipment was sent dry, the other half in moist condition). Collections were made near the time of sailing of the fast boats to New York, and shipments were placed in the steamer's refrigerator during transit.

The method of determining the presence of *Bartonella bacilliformis* in the insects was to inject a saline suspension of the crushed bodies intradermally, sometimes also intravenously, into monkeys (*Macacus rhesus*) and to make cultures of the blood at intervals of from one to four weeks later, irrespective of the occurrence of local lesions or fever. A given lot of insects was crushed in 0.9 per cent sodium chloride and injected intradermally into one or two sites on the shaved abdominal skin of two monkeys at least. Because of the differences in susceptibility of individual monkeys, duplicate tests were necessary.

The culture technique was the same as that used in earlier work on Carrion's disease (3). The blood was withdrawn from the monkey into an equal part of 2 per cent citrate in 0.9 per cent sodium chloride, and ascending dilutions in saline (1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000) were inoculated into the semisolid leptospira medium (4) in amounts of 0.2 cc. One tube of the medium was inoculated with a drop of the undiluted citrated blood. The cultures were kept at 30°C. By the end of a two to three week period the positive cultures can usually be picked out by their macroscopic appearance, but even microscopic examination may fail to reveal a positive culture, and subculture is often desirable (5). The growth may be so slight in the initial culture as to be easily mistaken for the haze which develops in a tube of sterile medium after standing at 30°C.

Although *Bartonella bacilliformis* was detected only in the phlebotomi, it is desirable to state that other insects collected (Shannon) in the verruga zone were inoculated into *rhesus* monkeys in the manner of the phlebotomi and the blood cultures carried out in the same way as for the latter. In no instance was *Bartonella bacilliformis* isolated in these cultures. A list of the insects with which no results were obtained follows:

Ticks.—Ornithodorus megnini (on burros), *Argas* sp.? (on burros, birds, bats), Tick larvae (genus and species?) on lizard.

Mites.—Tarsotomus sp. (on ground), *Trombidium* n.sp. (on ground), *Geckobia* sp. (on lizard), *Geckobiella* sp. (on lizard and geckos).

Lice.—Trichodectes ovis (on sheep).

Fleas.—Pulex irritans (on man), *Ctenocephalus canis* (on cat, dog, and man), *Rhopalopsyllus* (on dog and guinea pig).

Bedbugs.—Cimex lectularius.

Mosquitoes.—Anopheles pseudopunctipennis, Culex quinquefasciatus (fatigans).

Buffalo gnats.—Simulium escomeli, Simulium sp. (on burros).

Midges.—Forcipomyia utae, Forcipomyia townsendia.

Muscidæ.—Stomoxys calcitrans.

Hippoboscidae.—Melophagus ovinus (on sheep).

Streblidae.—3 genera, one species each (on vampire bats).

Note should be made of the fact that Townsend (2) regarded the lizard as the natural reservoir of the incitant of verruga peruana, for the reason that he detected intracorpuscular bodies in the blood cells, which he identified with Barton's rods. Hence the red mites (*Trombidium, Tarsotomus, Geckobia, Geckobiella*), some of which were obtained from geckos (*Phyllodactylus reisii*), as well as the blood of two geckos, were injected into monkeys. Cultures prepared from the blood of these monkeys remained sterile.

Phlebotomi of Verrugas Cañon.

One of us (Shannon) spent from March to July, 1928, in the Rimac verruga zone, Peru. As many varieties of insects as possible were collected (6) from this zone and sent to The Rockefeller Institute in New York to be tested upon monkeys. It is desirable to state that Townsend was the first to implicate phlebotomus with the transmission of verruga peruana. His studies, conducted between 1912 and 1914, led him to decide, on ecological and experimental grounds, that a species of gnat, later called *Phlebotomus verrucarum* Townsend, was the vector of the disease.

Our studies revealed three species of *Phlebotomus* in the verruga zone. Two of the species had a wide and the third a limited distribution only in the zone. It is significant that of the three, only the two which occurred in considerable numbers were found on inoculation to yield *Bartonella bacilliformis.*

In this paper brief descriptions only will be given of the three species, based upon the characters of the males. All three species belong to the subgenus *Brumptomyia* (França and Parrot), which may be described as follows:

Basal segment of the upper appendage of the male terminalia with a well defined sub-basal tuft of hairs on the inner surface; the distal appendage either with four well developed spines, the fifth one weak, or with five strong spines; median appendage simple, without spines, lower appendage unarmed. The abdominal hairs are suberect to erect; length of the upper branch of upper forked vein longer than the petiole preceding the fork.

Ph. noguchii and *Ph. peruensis* are described here for the first time (Shannon).

Key to the Males.

1. Distal segment of upper appendage with four well developed spines, the fifth (an apical one) being very slender and hair-like; petiole of upper forked

cell slightly longer than that section of the first longitudinal vein which overlaps the upper branch of the second vein..... *Ph. verrucarum* Townsend.

2. Distal segment with five well developed spines, both apical ones being equally strong.

(*a*) Distal segment with two submedian spines, a third located slightly distal of the middle of the segment, the fourth and fifth forming an apical pair; petiole of the upper forked cell much longer than that section of the first vein which overlaps the upper branch of the second vein. (Type locality, Verrugas Cañon, Department of Lima, Peru)

Ph. noguchii Shannon.

(*b*) Distal segment with two submedian spines, a subapical one and an apical pair; that section of the first vein overlapping the upper branch of the second is distinctly longer than the length of the petiole of the upper forked cell. (Type locality, Matucana, Department of Lima, Peru)

Ph. peruensis Shannon.

The females of *Phlebotomus peruensis* can be separated from those of *verrucarum* and *noguchii* by differences in the arrangement of the wing veins, but definite characters have not yet been found whereby the females of *verrucarum* and *noguchii* may be positively identified. Approximate identification of the females which were sent to New York for bacteriological study was made on the basis of (1) average differences in size, *verrucarum* being in general smaller than *noguchii*, and (2) habitat. All males found in houses proved to be *verrucarum*, hence all females found in-doors were assumed to belong to this species. All the *noguchii* males were found out-of-doors, in excavations and natural cavities, where they were three times as numerous as *verrucarum* males.

Before leaving this description, it may be well to bring together certain accepted facts with reference to the epidemiology of Carrion's disease and certain known habits of the phlebotomi. It is admitted that the disease is contracted only in certain limited areas in Peru, and that infection, with possibly rare exceptions, takes place only at night. This infection may be acquired indoors or in localities remote from human habitations and at any time of the year.

With these facts in mind, it would seem to follow that the insect vector must be (a) common blood sucker of man; (b) restricted to the verruga zone; (c) nocturnal in habit; (d) capable of breeding in varied localities, so that adults, which have restricted flight, may be everywhere present, and (e) active throughout the year.

All these conditions are fulfilled by phlebotomi and not, as far as determined, by other insects of the verruga zones.

Finally, it may be recorded that we (Shannon and assistant) safely

spent from 2 to 5 nights a week for 4 months in the verruga zone after taking adequate precaution to protect ourselves from bites of phlebotomi.

Experiments with Phlebotomi.

Eighteen special lots of phlebotomi, prepared and shipped as described, were inoculated into monkeys (Tilden and Tyler). The material in each instance was introduced intradermally at several sites on the shaved skin of the abdomen, and was also applied to a scarified area of the abdominal skin. Occasionally an intravenous injection was also made.

Lots 1, 2, 9, and 14 were pooled, ground in a mortar with 0.9 per cent saline solution, and injected, Apr. 25, 1928, into two monkeys (*Macacus rhesus* I-3 and I-4).

Lot 1, collected Mar. 9, 1928, out-of-doors in Matucana. 3 females and 4 males of *Ph. noguchii*, *Ph. verrucarum*, and *Ph. peruensis*.

Lot 2, collected Mar. 9, 1928, out-of-doors in Matucana. 2 females, 18 males, Phlebotomus, chiefly *noguchii*.

Lot 9, *Ph. verrucarum*, collected Mar. 20, 1928, in house in Verrugas Cañon. 40 females.

Lot 14, *Ph. verrucarum*, collected Mar. 26, 1928, in Verrugas Cañon. 24 females.

Lot 20. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, collected Apr. 9, 1928, consisted of about 20 females. A saline suspension of the crushed insects was injected May 31, 1928, into two monkeys (*M. rhesus* I-7 and I-8). I-8 also received 1 cc. of the suspension intravenously.

Lots 27 and 28. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, collected May 1 and May 8, 1928. 10 to 12 females. A saline suspension of the crushed insects was injected June 13, 1928, into two monkeys, *M. rhesus* I-17 and I-5.

Lots 29, 30, and 38. *Ph. verrucarum*, collected May 1 and May 8, 1928, 15 to 20 females. Saline suspension injected June 13, 1928, into *M. rhesus* I-16 and *M. rhesus* I-6.

Lots 40, 41, and 44. *Ph. verrucarum*, collected June 9, 11, and 18, 1928, both in houses and out-of-doors, in Verrugas Cañon. These lots comprised about 100 females. Saline suspension injected July 14, 1928, into *M. rhesus* I-26 and I-27. All the specimens which came in moist condition were covered with green mold.

Lots 39 and 45. *Ph. noguchii*, collected June 6 and June 19, 1928, out-of-doors in Verrugas Cañon. The number of insects was small (about 25 females), and the specimens which came in moist condition were covered with green mold. Saline suspension inoculated July 14, 1928, into *M. rhesus* I-28 and I-29.

Lots 42 and 46. *Ph. peruensis*, collected June 12 and 19, 1928, out-of-doors in Matucana. The specimens which came in moist condition were covered with green mold. 8 females. Saline suspension inoculated July 14, 1928, into *M. rhesus* I-30 and I-31.

Lot 43. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, collected June 12, 1928, out-of-doors in Matucana. Saline suspension inoculated Aug. 13, 1928, into two monkeys, I-33 and I-34.

Lot 51. *Ph. verrucarum*, collected during the last week in July, 1928. 6 females. Saline suspension inoculated Aug. 13, 1928, into *M. rhesus* I-37.

Lot 54. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, 8 to 10 females, collected during the last week in July out-of-doors in Verrugas Cañon. Saline suspension inoculated Aug. 14, 1928, into *M. rhesus* I-38 and I-39.

The first material inoculated, which contained all three species of Phlebotomus (Lots 1, 2, 9, and 14) yielded positive results.

Strain 1, from Lots 1, 2, 9, and 14.

M. rhesus I-3 and I-4, inoculated intradermally Apr. 25, 1928. No local lesions developed at the sites of inoculation. *M. rhesus* I-3 had a temperature of 104°F. on May 14, 19 days after inoculation, and blood was withdrawn on that day. *Bartonella bacilliformis* was obtained in culture from 1:10, 1:100, and 1:1,000 dilutions of the blood. The temperature reached 104°F. again several times, but blood culture was negative on May 29 and June 30. Blood was taken from *M. rhesus* I-4 at the same time as from I-3, but cultures remained negative.

Inoculation of Cultures from M. rhesus I-3.—*M. rhesus* I-14 and *M. rhesus* I-15 (Fig. 1) were inoculated June 5, 1928, with the 20 day culture of *Bartonella bacilliformis* obtained from the blood of *M. rhesus* I-3 and a 5-day subculture. The culture, which was, as usual, diluted with an equal part of saline for inoculation, was also applied to a scarified area on the abdominal skin. Tiny nodules were observed in *M. rhesus* 15 at the sites of intradermal inoculation on June 11 (16 days after inoculation), and 5 days later they were well developed, and one was excised[1] for examination and transfer. *Bartonella bacilliformis* was obtained from a 1:1,000 dilution of blood withdrawn June 16, and from a 1:100,000 dilution of the nodule suspension. By June 22 the scarified area showed small nodules. The photograph was taken 3 days later (Fig. 1). By June 28 the lesions had disappeared. *M. rhesus* I-14 had almost continuous high fever (104° to 106°F.) from June 8 to 28 and again from July 16 to 23, but no local lesions developed, and blood culture was negative 13, 31, and 55 days after inoculation.

The strain of *Bartonella bacilliformis* obtained from these first lots of phlebotomi was carried through two animal passages by direct transfer and has since been maintained by alternate generations in culture and

[1] All operations were carried out under ether anesthesia.

monkey. The usual course of verruga of moderate severity (7) has been observed in the animals (Fig. 8), with the exception of one monkey of the first passage (*M. rhesus* I-1), which had an unusually severe cutaneous reaction (Figs. 6, 7), not unlike that which had been induced in one of the monkeys (*M. rhesus* 18) of an early experiment (Noguchi (8)). This animal was acutely ill over a period of two weeks but recovered.

Histological study of the nodular tissue from I-15 and I-1, made by Dr. Henry R. Muller, shows the characteristic proliferation of endothelial cells and the formation of new capillaries. *Bartonella bacilliformis* was detected in some instances within the endothelial cells.

The second lot of phlebotomi tested, which probably consisted chiefly of *Ph. noguchii* but may have contained a few *verrucarum*, also yielded a strain of *Bartonella bacilliformis*.

Strain 2 from Lot 20.

M. rhesus I-7 and I-8 were inoculated May 31, 1928, intradermally, and I-8 received 1 cc. of the suspension in the left saphenous vein. The animals were bled on June 11 and again on June 30. The blood of I-8 yielded cultures of *Bartonella bacilliformis* in 1:100,000 dilution on June 11 and in 1:100 dilution on June 30; no cultures were obtained from the blood of I-7. Neither animal developed lesions at the sites of inoculation, but I-8 had almost continuous fever (104° to 105.2°F.) from June 4 to June 28.

Inoculation of Culture from M. rhesus I-8.—*M. rhesus* I-22 and I-23 were inoculated on June 22, 1928, intradermally, with a 10-day-old culture from the blood of *M. rhesus* I-8. The culture was also applied to a scarified area on the abdomen. The nodules showed at the sites of intradermal inoculation after about 10 days, and blood withdrawn after 2 weeks yielded cultures of *Bartonella bacilliformis* in 1:10,000 dilution. The nodule excised[1] on July 6 for examination and transfer yielded cultures in a 1:100 dilution. The lesions were considerably larger and more extensive in I-23 (Fig. 3), and the edema of the abdominal wall developed early and became marked. Regression of the lesions began about four weeks after inoculation and within four weeks recovery was practically complete. The course of disease was almost afebrile in both animals.

Further inoculations, first with nodular tissue from I-22 and I-23, and later with cultures from the blood of passage animals, showed that this strain of *Bartonella bacilliformis*, like Strain 1, was moderately virulent, inducing pronounced local lesions and moderate anemia.

One animal of the series died in 33 days, after an afebrile course of disease, during which moderately severe anemia had been observed.

Lots 27 and 28 (chiefly *Ph. noguchii*) and Lots 29, 30, and 38 (*Ph. verrucarum*) yielded negative results, as did also Lots 40, 41, 44, and 51 (*Ph. verrucarum*), Lots 42 and 46 (*Ph. peruensis*), and Lot 43 (*Ph. noguchii*).

From Lots 39 and 45, which probably consisted of *Ph. noguchii* alone, a third strain of *Bartonella bacilliformis* was obtained.

Strain 3, from Lots 39 and 45.

M. rhesus I-28 and I-29 were inoculated intradermally on July 14, 1928. Monkey I-28 showed no fever at any time, and blood cultures were negative 1, 2, and 3 weeks after inoculation. Monkey I-29 had fever (104.2° to 105.2°F.) 3 days after inoculation, which continued for a week with one day of remission. The blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:100 on four occasions, 7, 13, 23, and 31 days after inoculation, but there was no reaction at the sites of injection.

Inoculation of Cultures from I-29.—*M. rhesus* I-44 was inoculated intradermally and by scarification on Aug. 16, 1928, with a culture 14 days old from the blood of *M. rhesus* I-29. 1 cc. of the culture was also injected into the saphenous vein. Nodules developed in 2 weeks (Figs. 5 and 9), and the scarified area presented the characteristic miliary eruption. The blood was positive in a dilution of 1:10,000 at this time. From Sept. 7 to 19 there was marked fever (104° to 105.2°F.).

M. rhesus I-45 was inoculated at the same time and in the same manner as I-44. The intradermal nodules attained a diameter of only 0.5 cm., and the blood was positive in a dilution of 1:100. Fever existed (104.2° to 105.4°F.) from Sept. 10 to 13, was followed by two days of subnormal temperature, and death of animal on Sept. 16. Autopsy (Dr. Muller) revealed nothing abnormal except in the spleen, which contained numerous pale areas 2 to 3 mm. in diameter. Film preparations were negative for tubercle bacilli, and microscopic examination disclosed infarcts such as are found in the spleen in human (9) cases of Oroya fever, and in cases of the experimental disease (10).

Later passage of Strain 3 produced local lesions of very large size (2 to 3 cm. in diameter), but no unusual systemic effects.

Lot 54, which consisted chiefly, perhaps wholly, of *Ph. noguchii*, also yielded *Bartonella bacilliformis*.

Strain 4, from Lot 54.

M. rhesus I-38 was injected intradermally on Aug. 14, 1928, and intravenously (1 cc. of the saline suspension into the left saphenous vein). From Aug. 22 to

Aug. 29 the temperature was 104°F., but blood culture made Aug. 28 was negative. It was also negative on Sept. 10, but blood taken on Sept. 25, when the temperature was 104.2°F. yielded *Bartonella bacilliformis* in 1:10 and 1:100 dilutions after 13 days incubation. The intradermal mixtures produced no lesions. *M. rhesus* I-39, inoculated at the same time as I-38, and with the same material, showed a rise of temperature (104.2° to 104.8°F.) on three occasions, but blood cultures made on Aug. 28, Sept. 10, and Sept. 25 were negative.

Inoculation of Cultures from I-38.—M. rhesus I-58 was inoculated on Oct. 10, 1928, with 15-day culture from the blood of *M. rhesus* I-38. Small nodules appeared at the sites of intradermal injection after 7 days and were well advanced after 16 days (Fig. 5). The abdominal wall became oedematous and the area of scarification showed miliary nodules in addition to which three or four small eruptions arose outside the inoculated areas. Blood culture was positive in dilutions up to 1:10,000, 12 days after inoculation. The animal died on the 18th day, when the local lesions were still actively progressing. Histological examination of tissues by Dr. Muller revealed the characteristic zonal necrosis around the central vein in the liver, with extensive invasion by polymorphonuclear leucocytes. The spleen showed no lesions. The various skin nodules were histologically characteristic of verruga in the monkey.

Further inoculations with cultures of Strain 4 yielded similar results. In one animal (*M. rhesus* S-7) the local lesions reached large size.

The results of the inoculations are summarized in Tables I to V.

Exposure of Monkeys to Bites of Phlebotomi.

Six *rhesus* monkeys were exposed (Shannon) for several weeks to natural infection, three in an excavation in Verrugas Cañon, where *Ph. noguchii* was fairly common, and three in a house where *Ph. verrucarum* was abundant. These animals were brought to The Rockefeller Institute on Aug. 13. Blood withdrawn on three occasions failed to yield cultures of *Bartonella bacilliformis*, and only one of the animals failed to respond to subsequent inoculation of virulent cultures or passage virus. The result therefore was regarded as negative.

Immunity.

Seven of the monkeys which had developed verrucous lesions and blood infection with *Bartonella bacilliformis* following inoculation with the Phlebotomus strains and in which the lesions had regressed, were subsequently tested for immunity by reinoculation. Similar immunity tests were made on three monkeys which had received crushed

TABLE I.

Inoculations of Crushed Phlebotomi.

M. rhesus No.	Date 1928	Lot No.	Method of inoculation	Local lesions	Blood culture
I-3	Apr. 25	1, 2, 9, 14 *verrucarum* *noguchii* *peruensis*	Multiple intradermal Scarification	−	+
I-4	Same	Same	Same	−	−
I-7	May 31	20 *noguchii* (few *verrucarum?*)	Multiple intradermal Scarification	−	−
I-8	Same	Same	Same, also intravenous	−	+
I-16	June 13	29, 30, 38 *verrucarum*	Multiple intradermal Scarification	−	−
I-6	Same	Same	Same	−	−
I-17	Same	27, 28 *noguchii* (few *verrucarum?*)	Same	−	−
I-5	Same	Same	Same	−	−
I-26	July 14	40, 41, 44 *verrucarum*	Same	−	−
I-27	Same	Same	Same	−	−
I-28	Same	39, 45 *noguchii*	Same	−	−
I-29	Same	Same	Same	−	+
I-30	Same	42, 46 *peruensis*	Same	−	−
I-31	Same	Same	Same	−	−
I-33	Aug. 13	43 *noguchii* (few *verrucarum?*)	Same	−	−
I-34	Same	Same	Same	−	−
I-37	Same	51 *verrucarum*	Same	−	−
I-38	Same	54 *noguchii* (few *verrucarum?*)	Same, also intravenous	−	+
I-39	Same	Same	Same	−	−

TABLE II.

Strain 1, from Lots 1, 2, 9, 14 (Ph. verrucarum, Ph. noguchii, Ph. peruensis).

M. rhesus No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-14	June 5	Culture from I-3	Intradermal Scarification	−	−
I-15	Same	Same	Same	+++	+
		First passage			
I-18	June 16	Nodule susp. I-15	Same	++++	+
I-19	Same	Same	Same	++++	+
I-1	Same	Same	Same	++++	+
		Second passage			
I-12	July 6	Nodule susp. I-1	Same	++++	+
I-13	Same	Same	Same	+++	+
		Third passage (via culture)			
I-40	Aug. 16	14-day culture I-12	Same, also intravenous	++++	+
I-41	Same	Same	Same	++++	+

TABLE III.

Strain 2 from Lot 20 (Ph. noguchii—few verrucarum?).

M. rhesus No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-22	June 22	10-day culture from I-8	Intradermal Scarification	++++	+
I-23	Same	Same	Same	++++	+
		First passage			
I-24	July 6	Nodule susp. I-23	Same	++++	+
I-25	Same	Same	Same	+++	+
		Second passage (via culture)			
I-42	Aug. 16	14-day culture from I-24	Same, also intravenous	++++	+
I-43	Same	Same	Same	++++	+

TABLE IV.

Strain 3 from Lots 39 and 45 (Ph. noguchii).

M. rhesus No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-44	Aug. 16	14-day culture from I-29	Intradermal Scarification Intravenous	++++	+
I-45	Same	Same	Same	++++	+
		First passage			
I-55	Sept. 13	Nodule susp. I-44	Intradermal Scarification	+++	+
I-56	Same	Same	Same	++	−
I-34	Oct. 22	20-day culture from I-45	Same	+++	+
		Second passage (via culture)			
S-6	Dec. 15	25-day culture from I-34	Same	++++	+

TABLE V.

Strain 4 from Lot 54 (Ph. noguchii—few verrucarum?).

M. rhesus No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-58	Oct. 10	15-day culture from I-38	Intradermal Scarification	+++	+
		First passage (via culture)			
I-17	Nov. 5	14-day culture from I-58	Same	++++	+
		Second passage (via culture)			
S-7	Dec. 15	18-day culture from I-17	Same	++++	+

TABLE VI.

Immunity Tests.

M. rhesus No.	First inoculation				Immunity test			
	Date, 1928	Material inoculated	Local lesions	Blood culture	Date, 1928	Material inoculated	Local lesions	Blood culture
I-18	June 16	Nodule susp. I-15 (Str. 1)	++++	+	Sept. 13	Nodule susp. I-41 (Str. 1)	−	−
I-13	July 6	Same	++++	+	Same	Same	−	−
I-3	Apr. 25	Phlebotomi Lots 1, 2, 9, 14	−	+	Same	Same	++++	−
I-53 Control					Same	Same	++++	+
I-8	May 31	Phlebotomi Lot 20	−	+	Sept. 13	Nodule susp. I-43 (Str. 2)	++++	+
I-25	July 6	Nodule susp. I-23 (Str. 2)	++++	+	Same	Same	−	−
I-23	June 22	Blood culture I-8 (Str. 2)	++++	+	Same	Same	−	−
I-11	July 6	Nodule susp. P. 5*	++++	+	Same	Same	−	−
286	June 1	Culture P. 5*	++++	+	Same	Same	−	−
I-54 Control					Same	Same	+++	+
I-1	June 16	Nodule susp. I-15 (Str. 1)	++++	+	Sept. 26	Culture P. 5*	−	−
I-19	June 16	Same	++++	+	Same	Same	−	−
I-29	July 14	Phlebotomi Lots 39, 45	−	+	Same	Same	+++	+
I-57 Control					Same	Same	+++	+

* Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

phlebotomi without developing skin lesions which reacted as do previously untreated animals. Table VI summarizes the results.

Morphology.

No morphological or cultural differences could be detected between the Phlebotomus strains and the human strains of *Bartonella bacilliformis*. Cultures seven days old of the four strains, grown on horse blood agar slants, were stained to bring out the unipolar flagella (one to four) which are characteristic of *Bartonella bacilliformis* (Figs. 11, 13, 15, 17), the films being made on the same slide, in order that the stained preparations might be comparable. Cultures of the same age but grown on leptospira medium were used for similar comparative preparations which were stained by Gram's method, with fuchsin as the counterstain (Figs. 10, 12, 14, 16).

SUMMARY AND CONCLUSIONS.

With a view to determining the mode of infection in Carrion's disease, a study of the blood-sucking insects found in the districts of Peru where the disease prevails has been carried out, through the cooperation of The Rockefeller Institute and the Rockefeller Foundation. The material studied included ticks, mites, midges, lice, fleas, bedbugs, mosquitoes, buffalo gnats, horse-flies, "sheep ticks," 3 species of Streblidae, and 3 species of Phlebotomus, including *Phlebotomus verrucarum* Townsend and two new species which have been named *Phlebotomus noguchii* and *Phlebotomus peruensis*. The insects were collected without the use of chemicals, were prepared for transportation in such a manner as to prevent drying, and were shipped under conditions of refrigeration to New York, where they were inoculated into monkeys. The plan followed was to inject saline suspensions of the crushed insects intradermally into *rhesus* monkeys and to make cultures of the blood of the animals at intervals of 1 to 6 weeks after inoculation.

The only class of insects in which the presence of *Bartonella bacilliformis* could be detected were phlebotomi. No cutaneous lesions were induced in monkeys injected with the crushed insects, but in the case of four different lots of phlebotomi the blood of the animals so injected yielded cultures of *Bartonella bacilliformis* which produced typical verrucous lesions on inoculation into other monkeys.

The morphology and cultural characteristics of the Bartonella strains obtained from phlebotomi proved identical with those of strains

isolated from human blood and skin lesions. Monkeys which had recovered from infection with the phlebotomus strains resisted inoculation with a human strain of *Bartonella bacilliformis*, and, conversely, monkeys which had passed through an infection induced by the human strain resisted inoculation with the strains obtained from phlebotomi.

The experimental observations described in this paper lead us to conclude that certain phlebotomi act as insect vectors of Oroya fever and verruga peruana. The phlebotomi which have been shown quite certainly to carry the *Bartonella bacilliformis* are those of the species *Phlebotomus noguchii*. *Phlebotomus verrucarum* is also probably a vector, while *Phlebotomus peruensis* remains doubtful in this respect.

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EXPLANATION OF PLATES.

PLATE 45.

FIG. 1. Cutaneous lesions induced in *M. rhesus* I-15 by Phlebotomus Strain 1. Photograph taken 20 days after inoculation. One intradermal nodule had been excised 2 days previously.

FIG. 2. The appearance of the lesions in *M. rhesus* I-1, a Strain 1 first passage animal, 18 days after inoculation. The sacrified area already shows characteristic minute nodules. All the lesions reached considerable size (Figs. 6 and 7).

FIG. 3. Strain 2. Early culture lesions (two weeks after inoculation) in *M. rhesus* I-23.

FIG. 4. *M. rhesus* I-44, 21 days after inoculation with cultures of Strain 3 from Lots 39 and 45 of *Ph. noguchii*.

FIG. 5. *M. rhesus* I-58, 16 days after inoculation with Strain 4 phlebotomus cultures. The eruption was more general and the edema extensive in this animal. Death occurred 3 days after the photograph was made.

PLATE 46.

FIGS. 6 AND 7. Late lesions in *M. rhesus* I-1 (Strain 1) as they appeared 31 days after inoculation. The most pronounced lesion occurred at the scarification site (center).

FIG. 8. *M. rhesus* I-19, 29 days after inoculation in the same way and at the same time as *M. rhesus* 1.

FIG. 9. *M. rhesus* I-44, 21 days after inoculation with cultures of *Phlebotomus noguchii* Strain 3.

PLATE 47.

Magnification × 1,000.

FIG. 10. Phlebotomus Strain 1, from Lots 1, 2, 9, 14. Gram's stain, counterstained with saturated alcoholic solution of fuchsin.

FIG. 11. Same, stained for flagella, by a combination of Zettnow's mordant and Fontana's ammoniac silver solution.

FIG. 12. Phlebotomus Strain 2, from Lot 20. Gram's stain, counterstained with fuchsin.

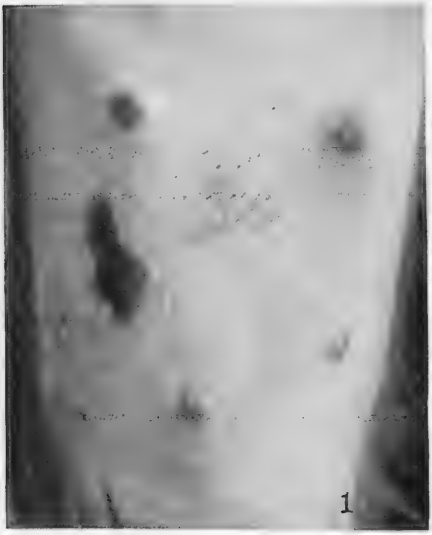
FIG. 13. Same, Zettnow-Fontana flagella stain.

FIG. 14. Phlebotomus Strain 3, from Lots 39 and 45. Gram's stain, counterstained with fuchsin.

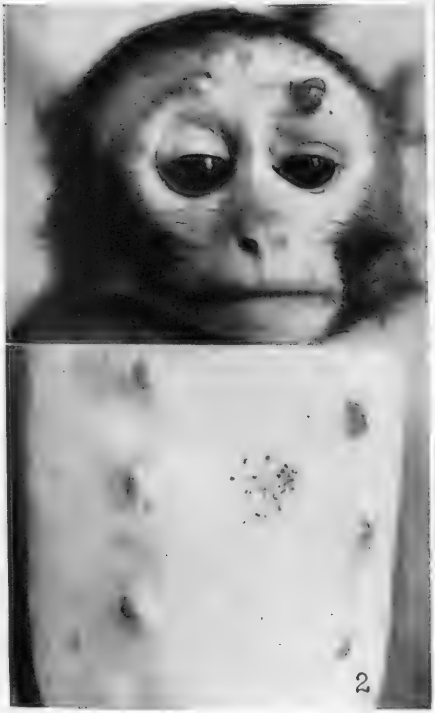
FIG. 15. Same, Zettnow-Fontana flagella stain.

FIG. 16. Phlebotomus Strain 4, from Lot 54. Gram's stain, counterstained with fuchsin.

FIG. 17. Same, Zettnow-Fontana flagella stain.

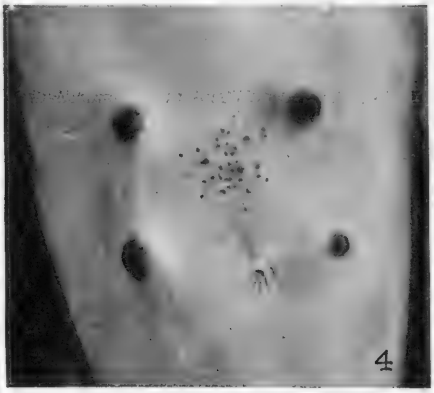


M. rhesus I–15. Strain 1.
20 days after inoculation.

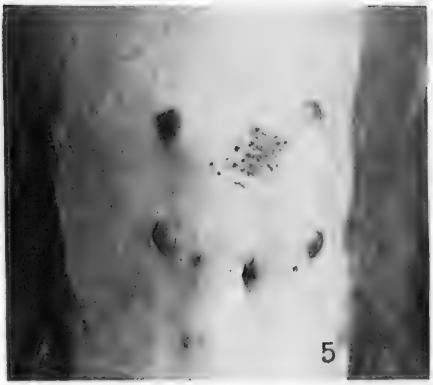


M. rhesus I–1. Strain 1.
18 days after inoculation.

M. rhesus I–23. Strain 2.
14 days after inoculation.



M. rhesus I–44. Strain 3.
21 days after inoculation.



M. rhesus I–58. Strain 4.
16 days after inoculation.

(Noguchi *et al.*: Etiology of Oroya fever. XIV.)

M. rhesus I-1, Strain 1,
31 days after inoculation.

M. rhesus I-19, Strain 1,
29 days after inoculation.
Scarified area in center.

M. rhesus I-1, Strain 1, 31 days after inoculation. The largest lesion arose on the area inoculated by scarification (center).

M. rhesus I-44, Strain 3,
21 days after inoculation.
Scarified area in center.

(Noguchi *et al.*: Etiology of Oroya fever. XIV.)

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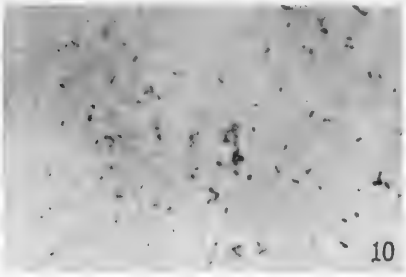
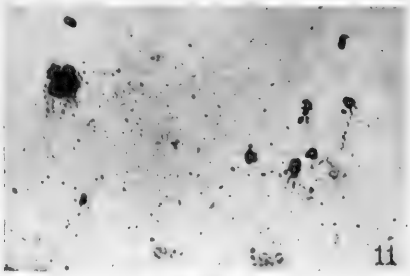
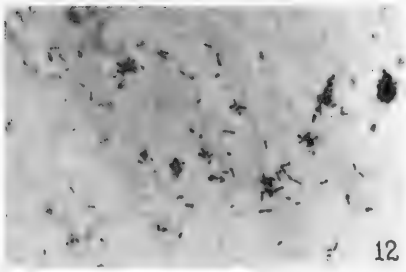


Strain 1.

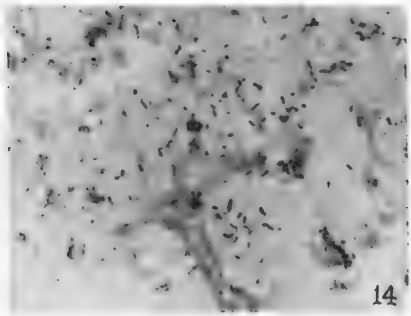


Strain 1.

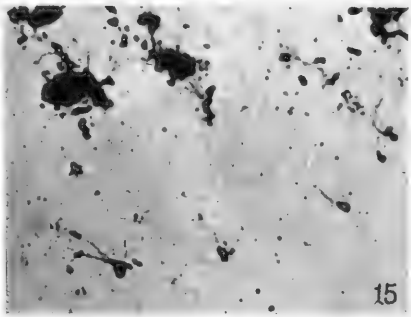


Strain 2.

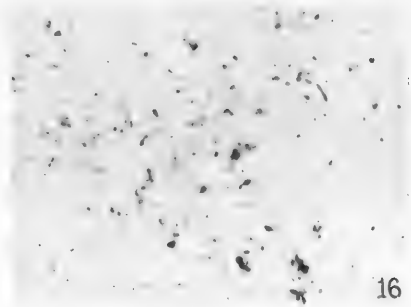
Strain 2.



Strain 3.

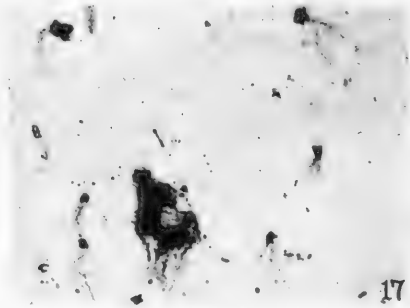


Strain 3.



Strain 4.

Gram, counterstained with fuchsin.



Strain 4.

Zettnow-Fontana combination stain.

× 1,000.

(Noguchi *et al.*: Etiology of Oroya fever. XIV.)

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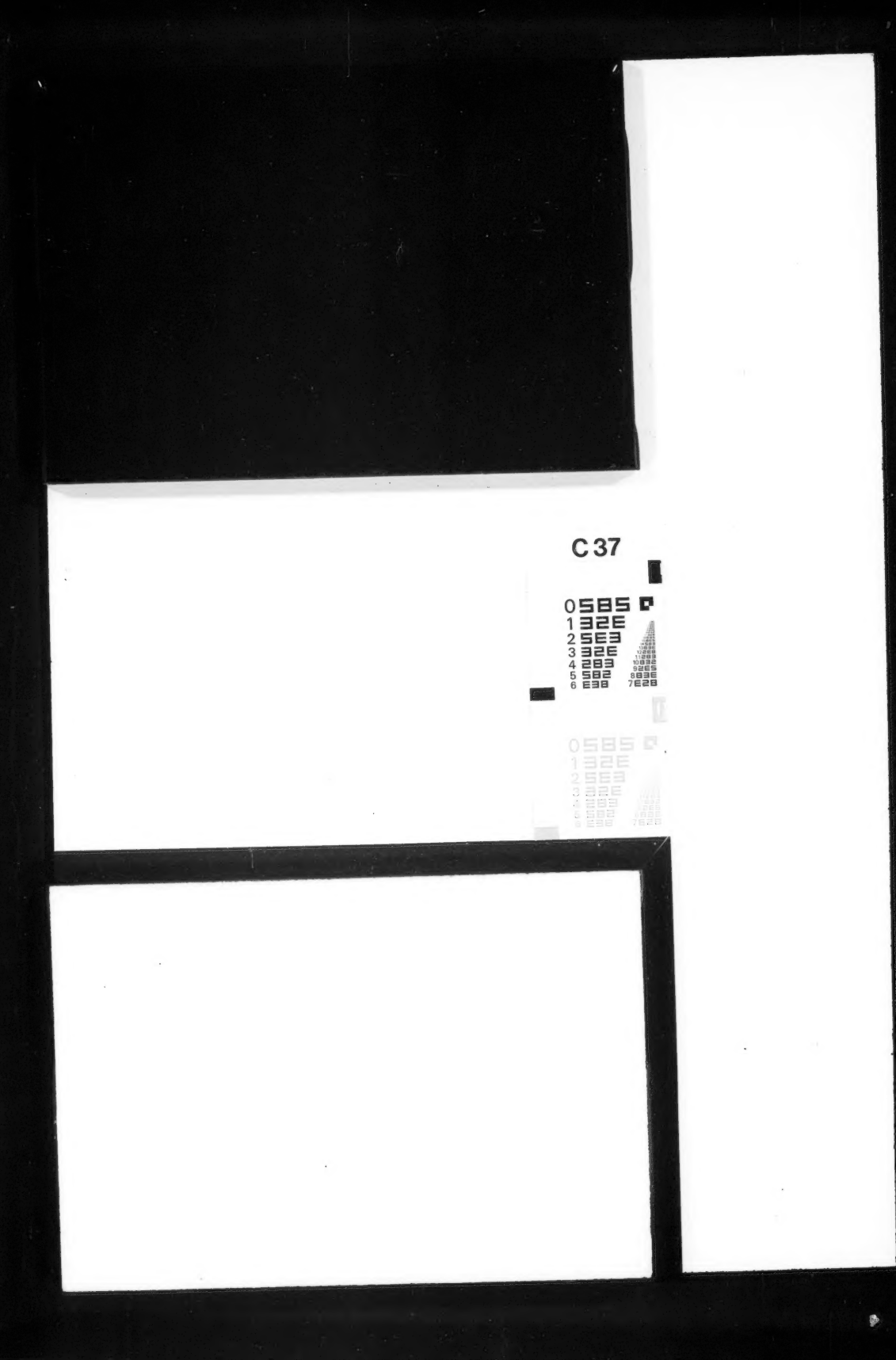
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